REMARKS

Claims 1, 3-6 and 8 are pending in the application. Applicant thanks the Examiner for deeming claims 1 and 3-5 to be allowable. Claim 6 is amended to improve clarity. New claims 9 and 10 are added. Support for new claims 9 and 10 is found in the published application at least at paragraphs [0012] and [0050]. Upon entry of the above-made amendments, claims 1, 3-6 and 8-10 will be pending in the application. Applicant reserves the right to prosecute the subject matter of any canceled claims, or any unclaimed subject matter, in a related application.

No new matter has been added by these amendments. Entry of the foregoing amendments and consideration of the following remarks are respectfully requested.

The Rejection Under 35 U.S.C. § 112, First Paragraph, For Lack of Enablement, Should Be Withdrawn

The Examiner has rejected claims 6 and 8 under 35 U.S.C. § 112, first paragraph, as allegedly nonenabled. Applicant traverses as follows.

Analysis of enablement requires a determination of whether the "disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention." MPEP 2164.01 at page 2100-178. One skilled in the art is presumed to use the information available to him in attempting to make or use the claimed invention. *See Northern Telecom, Inc. v. Datapoint Corp.*, 908 F.2d 931, 941 (Fed. Cir. 1990). The standard for determining whether a claim is enabled or not is whether it requires undue experimentation to practice. *Id.*; *see also Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916); *In re Wands*, 858 F.2d 731, 737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). In *Wands*, the Federal Circuit outlined several non-exclusive factors to consider in a determination of whether claims were enabled, including: A) the breadth of the claims; (B) the nature of the invention; (C) the state of the prior art; (D) the level of one of ordinary skill; (E) the level of predictability in the art; (F) the amount of direction provided by the inventor; (G) the existence of working examples; and (H) the quantity of experimentation needed to make or use the invention based on the content of the disclosure. In *Wands*, the Federal Circuit held that a particular method requiring substantial experimentation was nonetheless enabled, because the experimentation was routine.

The use of the peptide vector of the invention, as claimed in claims 6 and 8, is enabled because Applicant teaches how to construct specific peptides that mimic viral entry

sequences, how to link these peptides to a polynucleotide of interest, how to introduce the peptide vector/polynucleotide combination into an individual (*e.g.*, mouse), and how to determine whether the polynucleotide of interest has been expressed in cells in that individual. Because the use of the claimed peptide vector is quite simple, and because the state of polynucleotide expression art is very well-developed, a person of skill in the art, reading the specification, would enjoy a reasonable expectation of success in using the peptide vector to transfer and express a polynucleotide *in vivo*. *See*, *e.g.*, Examples 1-4.

The Examiner bases nonenablement primarily on three assertions: (1) that the invention, as exemplified, "does not overcome the problem of gene delivery to targeted cells"; (2) that the invention, as exemplified, "does not solve the problem of transient expression"; and (3) that the experiment described in the Examples "fails to show that the transferred protein was translated so that the desired protein would be expressed at sufficient levels to produce a desired function in the target cells." Office Action at page 3. Applicant addresses each of these assertions in turn.

The Peptide Vector Delivers a Polynucleotide to Target Cells In Vivo

First, the present specification does overcome the problem of polynucleotide, *e.g.*, gene, delivery to target cells, and does so *in vivo*, as described in the Examples.[1] The Examiner, in the interview of November 15, 2005, suggested that claims 6 and 8 would be allowable if restricted to *in vitro* methods. Thus, polynucleotide delivery to cells, *per se*, appears to be supported by the specification. Moreover, Example 4 demonstrates that a polynucleotide was actually delivered into cells *in vivo*, as evidenced by PCR detection of GFP-specific mRNA. In Example 4, a peptide vector containing a GFP-encoding sequence was injected intravenously into a male mouse. Page 11, lines 17-18. The peptide vector, constructed according to Examples 1-3, comprises peptides having sequences that mimic viral sequences that enable viral fusion with a cell membrane; thus, the peptide vector is designed to enter a cell in the same manner as a virus. *See*, *e.g.*, page 6, lines 5-10. Continuing in Example 4, the mouse was sacrificed six days later, and mRNA was extracted

---

[1] To the extent the Examiner uses "targeted cells" to mean one or several specific cell populations *in vivo*, Applicant points out that the peptide vector is designed to be a nonspecific polynucleotide delivery vehicle. *See*, *e.g.*, page 5, lines 7-8, page 6, lines 3-4, and Example 4. Nothing in Verma, or the other references cited by the Examiner, suggests that a non-specific polynucleotide delivery system would be unpredictable, or otherwise nonenabled, simply because of its non-specificity.

from brain and muscle. Page 11, lines 19-20. The mRNA was reverse-transcribed, and PCR was performed on the resulting cDNA using GFP-specific primers. Page 12, lines 1-15. PCR showed that GFP was efficiently expressed in brain and muscle (FIG. 2).

Because mice contain no native GFP-encoding sequence, the GFP mRNA *must* have originated with the GFP-encoding sequence of the peptide vector of the invention. It follows that the peptide vector of the invention *must* have delivered the GFP-encoding sequence to target cells in the mouse. Example 4 is, therefore, far more than "an example wherein mRNA levels of GFP is measured using GFP specific primers," as characterized by the Examiner. Office Action, page 3. The peptide vector of the invention, therefore, *does* overcome the problem of gene delivery to target cells *in vivo*.[2]

Transient Expression is Not a Barrier to Enablement

Second, transient expression, in and of itself, does not weigh against enablement. To the extent the invention encompasses gene therapy, it encompasses a method of treatment of an individual in, for example, a medical context. The field of medicine is replete with examples of therapies that act only transiently. A simple example is a non-steroidal anti-inflammatory drug ("NSAID"), such as ibuprofen. Once taken, the NSAID exerts its effect for 4-24 hours, after which its effect is lost. It is a given that NSAIDs are extraordinarily useful compounds, despite their transient effect. Another example is the plethora of cancer therapies available. Most, if not all, are only transiently effective, in that they must be taken regularly in a dosing regimen to have a measurable effect, and in that they frequently work against the disease only for a period of weeks or months. Despite these problems, however, they are viewed by the medical profession as valuable therapeutic tools. In the same way, an individual would benefit from gene therapy would benefit from the a polynucleotide carried by the peptide vector of the invention, even if the measurable effects of the polynucleotide are similarly transient. Certainly, stable, long-term expression is an ultimate goal for gene therapy research, much as a cure for cancer is an ultimate goal for cancer research. At the present time, however, it is more the rule, not the exception, that highly useful therapies are transiently-effective.

---

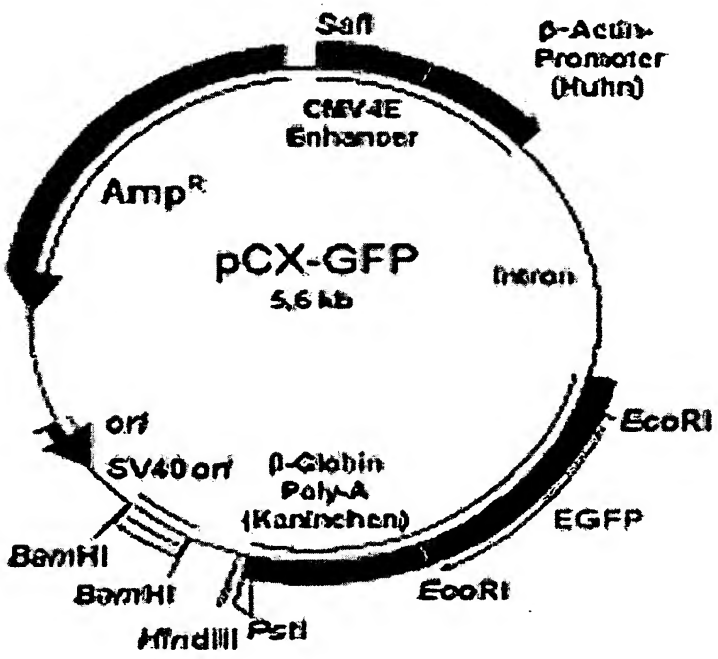
[2] Applicant respectfully notes that the Examiner has not explained how the GFP mRNA, described in Example 4 as being collected from mouse brain and muscle, could *not* have been delivered to targeted cells by the peptide vector of the invention.

Transient expression of a polynucleotide carried by the peptide vector, to the extent it occurs, is not relevant to enablement. A therapy is not made more unpredictable simply because it is transiently-effective. Likewise, a person of skill in the art would not be subjected to undue experimentation simply because a peptide vector-borne polynucleotide is transiently expressed. The use of the peptide vector, given any transient expression, would simply be subject to routine dosing experimentation and optimization, as would any other therapeutic. Nothing in Verma (*Nature* 389:239-242 (1997)), or the other references cited by the Examiner, suggests otherwise.

The Working Examples Demonstrate Expression of a Protein From the Vector

Third, the specification, including Examples 1-4, provides enough information such that a person of skill in the art would believe that the transferred polynucleotide, the GFP-encoding sequence, was translated so that the encoded protein would be expressed at a sufficient level to have an effect *in vivo*. As noted above, and as described in the Examples, Applicant detected GFP mRNA in tissues of experimental mice, which produce no native GFP.

The GFP-encoding sequence used in Example 4, carried by the peptide vector, and expressed in the experimental mouse, was obtained from plasmid pCX-GFP, a diagram of which is reproduced below:



The plasmid was digested with *Bam*HI and *Sal*I. As shown above, this *Bam*HI-*Sal*I fragment comprises the GFP-encoding sequence (designated EGFP[3]), a β-actin promoter, the CMV-IE enhancer, and β-globin poly-A sequence. Thus, the polynucleotide used in the Examples of the present specification was designed specifically to express the GFP-encoding sequence in mammalian cells. Given this design, a person of skill in the art would reasonably believe that, as described in Example 4, the GFP-encoding polynucleotide in the peptide vector was expressed and that significant amounts of GFP were produced.

Moreover, GFP, including EGFP, is one of the most widely-known and accepted experimental markers; *see, e.g.*, Bierhuizen *et al.*, "Enhanced Green Fluorescent Protein as Selectable Marker of Retroviral-Mediated Gene Transfer in Immature Hematopoietic Bone Marrow Cells," *Blood* 90(9):3304-3315 (1997); *see also* Yamanouchi *et al.*, "Identification of Skeletal Muscle Satellite Cells by Transfecting EGFP Driven by Skeletal α-Actin Promoter," *J. Vet. Med. Sci.* 62(11):1213-1216 (2000). Copies of these references are provided for the Examiner's convenience. Because the sequences included within the exemplified peptide vector were designed to express GFP, a person of skill in the art would understand that the mouse brain and muscle tissue, from which the GFP mRNA was obtained in Example 4, produced sufficient quantities of green fluorescent protein to be detectable in a fluorescence assay. No actual fluorescence data is needed to demonstrate that protein production has taken place.[4]

In addition, the art of protein expression from polynucleotides, including the provision of appropriately-oriented promoter, enhancer, and polyadenylation sequences that facilitate expression, was very well-developed at the time of filing. Thus, once a protein coding sequence is introduced into a cell, regardless of the method of introduction into the cell, the expression of that sequence, and the production of useful quantities of protein, was, at that time, routine to those of skill in the art. A person of skill in the art, therefore, could easily and routinely modify the peptide vector described in the specification to enable the

---

[3] EGFP = Enhanced Green Fluorescent Protein, a version of GFP that produces enhanced fluorescence compared to native GFP.

[4] Applicant's representative had, in the November 15, 2005 interview, discussed the possibility of submitting a Declaration to address this issue. Ultimately, it was decided that the specification, in combination with protein expression methods generally known to those of skill in the art, were sufficient to enable the methods of using the peptide vector of the invention *in vivo*.

expression *in vivo* of a wide variety of protein-coding sequences. A person of skill in the art, therefore, having the specification and knowledge of gene expression technology in hand, would, at the time of filing, have enjoyed a reasonable expectation of success in using the peptide vector to transfer a polynucleotide to cells *in vivo*, and in having the polynucleotide expressed in those cells.

With respect to Choi (*Vet. Immun. Immunopathol.* 103:223-233 (2005)), cited by Applicant in the Amendment of March 28, 2005, Applicant is aware that Choi was published after the filing date of the present application. The purpose of citing Choi, however, was to demonstrate that the specification, *at the time of filing*, was enabling. Choi, as explained in the previous Amendment, shows construction of a peptide vector using the same peptides as described in the present application, and using an expressible polynucleotide, as in the present application. Choi, like the present application, shows administration to an animal, and evidence of expression of the polynucleotide. As such, Applicant cites Choi for that which was already disclosed in the present application, and to demonstrate that the methods of the present application did not require undue experimentation, and provided a reasonable expectation of success. Choi is, therefore, evidence that the use of the peptide vector *in vivo*, as described in the present specification, is effective and, therefore, that the present specification is enabling.

Applicant therefore respectfully submits that the specification enables the pending claims because (1) the specification demonstrates gene delivery to target cells, and expression, in a model animal; (2) transiently-effective therapeutics are widely accepted in the field of medicine, and transcience of an effect does not indicate unpredictability; (3) the GFP expression system used in the Examples is one that persons of skill in the art would reasonably expect to produce significant amounts of GFP, and other expressible polynucleotides would be expected to work as well; and (4) a person of skill in the art, having the specification's teachings in hand, would be able to practice the *in vivo* method as claimed in claims 6 and 8 without undue experimentation, and with a reasonable expectation of success.

CONCLUSION

Applicant respectfully requests entry of the foregoing remarks into the file of the above-identified application. Applicant believes that all the pending claims are in condition for allowance. Withdrawal of the Examiner's rejections and allowance of the application are respectfully requested.

Respectfully submitted,

Date: December 15, 2005

Lawrence S. Graham Reg. No. 49,020
for: Anthony M. Insogna Reg. No. 35,203
JONES DAY
222 East 41st Street
New York, New York 10017-6702
Phone: (212) 901-9028

RAPD COMMUNICATION

Enhanced Green Fluorescent Protein as Selectable Marker of Retroviral-Mediated Gene Transfer in Immature Hematopoietic Bone Marrow Cells

By Marti F.A. Bierhuizen, Yvonne Westerman, Trudi P. Visser, Wati Dimjati, Albertus W. Wognum, and Gerard Wagemaker

The further improvement of gene transfer into hematopoietic stem cells and their direct progeny will be greatly facilitated by markers that allow rapid detection and efficient selection of successfully transduced cells. For this purpose, a retroviral vector was designed and tested encoding a recombinant version of the *Aequorea victoria* green fluorescent protein that is enhanced for high-level expression in mammalian cells (EGFP). Murine cell lines (NIH 3T3, Rat2) and bone marrow cells transduced with this retroviral vector demonstrated a stable green fluorescence signal readily detectable by flow cytometry. Functional analysis of the retrovirally transduced bone marrow cells showed EGFP expression in in vitro clonogenic progenitors (GM-CFU), day 13 colony-forming unit-spleen (CFU-S), and in peripheral blood cells and marrow repopulating cells of transplanted mice. In conjunction with fluorescence-activated cell sorting (FACS) techniques EGFP expression could be used as a marker to select for greater than 95% pure populations of transduced cells and to phenotypically define the transduced cells using antibodies directed against specific cell-surface antigens. Detrimental effects of EGFP expression were not observed: fluorescence intensity appeared to be stable and hematopoietic cell growth was not impaired. The data show the feasibility of using EGFP as a convenient and rapid reporter to monitor retroviral-mediated gene transfer and expression in hematopoietic cells, to select for the genetically modified cells, and to track these cells and their progeny both in vitro and in vivo.

RETROVIRAL VECTORS based on the Moloney murine leukemia virus (MoMLV) backbone are widely studied as vehicles for gene transfer into hematopoietic cells based on their potential for highly efficient transduction and integration in the host genomic DNA.[1-6] Significant restrictions of these vectors are the dependence on cell division for successful integration[7,8] and an inconsistent expression level of vector-encoded proteins in target cells.[9,10] These issues are currently addressed in retroviral-mediated gene transfer procedures for hematopoietic stem cells, a small population of predominantly noncycling cells.[11,12] However, determination of transduction efficiency and expression in these cells has been difficult and relied mostly on studying the presence of the transgene in more differentiated cell types after outgrowth in in vitro cell culture assays or in vivo repopulating assays. Therefore, an important tool to improve gene transfer procedures is the availability of markers that allow rapid detection of the transferred gene in immature hematopoietic cells and enrichment of the transduced cell populations.

Retroviral vectors encoding selectable reporter molecules have been used to study the efficiency of transduction in immature hematopoietic cells and the long-term presence of the transgene in their progeny after transplantation in recipient animals. The encouraging results obtained for murine hematopoietic stem cells[13-15] have stimulated the use of recombinant retroviruses for the genetic modification of the corresponding cells of larger animals. However, comparably high transduction efficiencies of long-term repopulating cells of canine, rhesus monkey, or human origin have not been achieved yet.[16-18] Nevertheless, the neomycin phosphotransferase gene (neo) has proven to be particularly useful in tracking retrovirally transduced human hematopoietic cells both in vitro and in vivo, and in the identification of cells capable of causing leukemic relapse after autologous bone marrow (BM) transplantation.[19-21] For the enrichment of immature and transduced hematopoietic cells this marker gene is less suitable, mostly because a selective advantage is only obtained after time-consuming exposure to toxic drug concentrations under in vitro culture conditions that stimulate cell division and terminal differentiation. The bacterial β-galactosidase gene (lacZ) has also been used as a selectable reporter molecule for hematopoietic cells,[22-25] but the relatively high endogenous β-galactosidase activity in some cell types and the requirement for transporting fluorogenic substrates across the cell membrane while maintaining cell viability have limited its application.

Recently, several groups have independently studied transduction of hematopoietic cells with retroviral vectors encoding marker molecules expressed at the cell surface, thus allowing for the direct identification of transduced cells with specific antibodies.[26-29] These studies included the successful transduction of $CD4^+$ and $CD8^+$ T lymphocytes with the gene for the human low-affinity nerve growth factor receptor[29] and transduction of human $CD34^+CD38^+$ and the more immature $CD34^+$ $CD38^{low}$ BM cells by a retroviral vector encoding the murine heat-stable antigen.[27] Such approaches offer as potential advantages the rapid study of a variety of parameters on gene transfer efficiency of immature hematopoietic cells. Prerequisites for this type of procedure evidently are the absence or low-level expression of the reporter molecule in the target cells and the availability of antibodies specific for the marker.

From the Institute of Hematology, Erasmus University Rotterdam, Rotterdam, The Netherlands.

Submitted June 11, 1997; accepted August 11, 1997.

Supported in part by the Netherlands Cancer Foundation Koningin Wilhelmina Fonds, the Dutch Organization for Scientific Research NWO, the Royal Netherlands Academy of Arts and Sciences, and Contracts of the Commission of the European Communities.

Address reprint requests to Marti F.A. Bierhuizen, PhD, Institute of Hematology, Erasmus University Rotterdam, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/97/9009-0052$3.00/0

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has recently been introduced as a promising reporter for monitoring gene transfer and expression in eukaryotic cells.[30] Purified wildtype GFP emits green light after illumination with UV light.[31-33] Except for the presence of oxygen and excitation with blue light, it requires no further exogenous molecules to fluoresce and, therefore, appears to be superior to other markers used for the selection of viable cells thus far. However, because wildtype GFP has only a minor absorption maximum at 470 nm it produces only a relatively weak green fluorescence signal when excited by a standard fluorescence-activated cell sorter (FACS) or a fluorescence microscope. Several GFP variants have been created with maximum excitation peaks at 490 nm which are better suited for detection of expression by these methods.[34-37] Recently, the generation of stable producer cell lines for retroviral vectors encoding GFP variants has been independently reported by a number of groups and the powerful potential of GFP as a selectable marker of retroviral-mediated gene transfer was demonstrated in murine and human cell lines.[38-41] In a direct comparison of the relative fluorescence intensities of retroviral vectors encoding distinct GFP variants we showed by FACS analysis that EGFP, a GFP variant enhanced for high-level expression in mammalian cells, provided the highest fluorescence intensity in murine fibroblasts.[41] The present study was directed at the use of EGFP for the identification and selection of retrovirally transduced primary murine BM cells and tracking their progeny after transplantation in recipient mice.

MATERIALS AND METHODS

Animals and cells. Animals used in these experiments were bred in the Central Animal Department of the Erasmus University (Rotterdam, The Netherlands) and maintained under specific pathogen-free conditions. For retroviral transduction experiments, BM cells were used from 8-week-old female (C57BL × CBA)F1 hybrid (BCBA) mice. Mice used as recipients in the colony-forming unit-spleen (CFU-S)/marrow repopulating ability (MRA) assays were ≥12-week-old female BCBA mice. Murine BM cells were prepared by flushing femora with an enriched version of Dulbecco's modified Eagle's medium (DMEM; GIBCO, Gaithersburg, MD). The DMEM used for this study was enriched with L-alanine (25 mg/L), L-asparagine (50 mg/L), L-aspartic acid (30 mg/L), L-cysteine (70 mg/L), L-glutamic acid (75 mg/L), L-proline (40 mg/L), sodium pyruvate (110 mg/L), vitamin B_{12} (25 μg/L), and biotin (30 μg/L) (all from Sigma, St Louis, MO). Murine BM cells were cultured in this enriched DMEM further supplemented with 1% (wt/vol) BSA (Fraction V; Sigma), 0.3 mg/mL human transferrin, 0.1 μmol/L sodium selenite, 1 mg/L nucleosides (cytidine, adenosine, uridine, guanosine, 2′-deoxycytidine, 2′-deoxyadenosine, thymidine, and 2′-deoxyguanosine; Sigma), 0.1 mmol/L β-mercaptoethanol, 15 μmol/L linoleic acid, 15 μmol/L cholesterol, 1 μmol/L hydrocortison, and 1 μmol/L isoprotenol.[42-44] The ecotropic and amphotropic packaging cell lines GP+E-86[45] and GP+*env*Am12,[46] respectively, as well as Rat2[47] and NIH 3T3 fibroblasts were cultured in DMEM supplemented with 10% (vol/vol) newborn calf serum. Media for all cultures routinely included 100 U/mL of penicillin and 100 μg/mL of streptomycin. The cultures were maintained at 37°C with 10% CO_2/90% air in a humidified atmosphere.

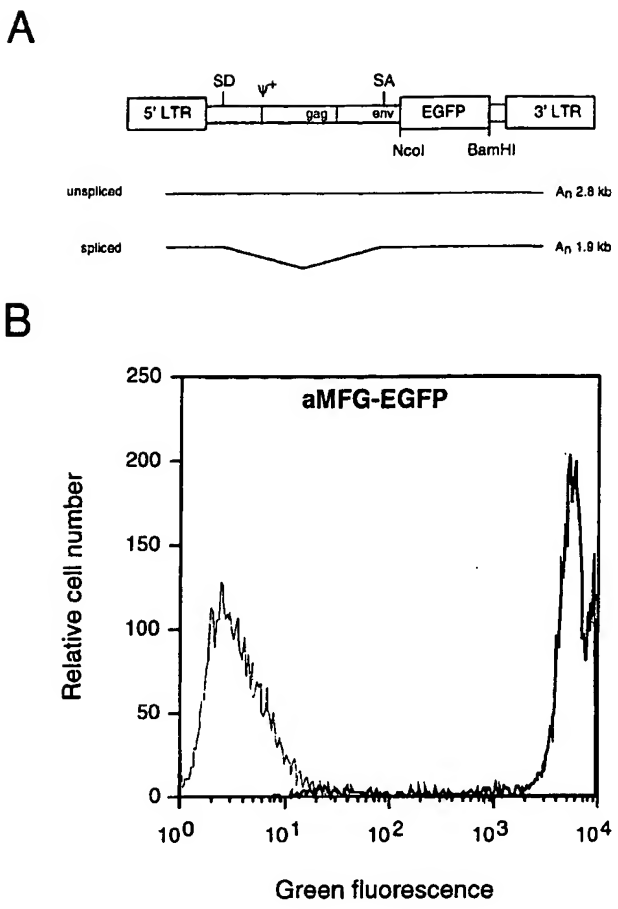
Retroviral vectors. For all experiments, retrovirus was used derived from the MFG retroviral vector[48] provided by Dr R.C. Mulligan (Whitehead Institute for Biomedical Research, Cambridge, MA). To construct MFG-EGFP, a 726-bp fragment encoding EGFP was inserted into the *Nco I/Bam*HI sites of MFG using standard procedures. The EGFP-specific fragment was generated by polymerase chain reaction (PCR) using two synthetic oligonucleotides (Pharmacia, Uppsala, Sweden) as primers and pEGFP-C1 (Clontech Laboratories Inc, Palo Alto, CA) as template. The 5′-primer, 5′-TTTAAGCTTGCCACCATGGTGAGCAAGGGCGAG-3′, corresponds to nucleotides 607 to 630 of pEGFP-C1 and contains the start codon embedded into an *Nco* I site. The 3′-primer, 5′-TTTGGATCCTTACTTGTACAGCTCGTCCATGCC-3′, corresponds to nucleotides 1309 to 1330 of pEGFP-C1 and contains downstream of the termination codon a *Bam*HI site. In this way, EGFP expression is controlled by the MoMLV long terminal repeat (LTR) with its start codon placed exactly at the position of the MoMLV envelope ATG.[48]

Retrovirus production. GP+E-86 ecotropic retroviral packaging cells were cotransfected with both the MFG-EGFP vector and pSV2neo using calcium phosphate precipitation, and subsequently cultured in the presence of G418 (500 μg/mL of active drug) to select for a polyclonal population of stable transfected cells. Cells displaying the brightest green fluorescence were cloned by single-cell FACS selection and expanded. The viral titer of the clonal ecotropic MFG-EGFP producer (eMFG-EGFP) used for this study was in the order of 10^5 infectious particles per milliliter as assessed by the transfer of EGFP expression to NIH 3T3 fibroblasts. Clonal amphotropic retroviral producer cell lines were obtained by transduction of GP+*env*Am12 cells with supernatants containing ecotropic retrovirus followed by single-cell FACS selection on the basis of green fluorescence expression as mentioned above. The viral titer of the clonal amphotropic MFG-EGFP producer (aMFG-EGFP) used for this study was in the order of 10^6 infectious particles per milliliter. Absence of replication-competent virus was verified by failure to transfer green fluorescence protein expression from a transduced cell population to NIH 3T3 fibroblasts.

RNA blot analysis. Total RNA was purified from the aMFG-EGFP producer cell line and, as a control, GP+*env*Am12 cells as described[49] and resolved by 2.2 mol/L formaldehyde/1.2% agarose gel electrophoresis. After blotting to a nylon membrane, RNAs were visualized by hybridization with a ^{32}P-labeled EGFP-specific cDNA.

Retroviral transduction of cell lines and BM cells. Supernatants containing recombinant retrovirus were generated by culturing approximately 80% confluent producer cells for 16 to 24 hours in the appropriate culture medium. The culture supernatant was subsequently procured and passed through a 0.45-μm filter. For transduction of Rat2 and NIH 3T3 fibroblasts supernatants containing aMFG-EGFP retrovirus were supplemented with hexadimethrine bromide at 4 μg/mL (Sigma) and layered onto subconfluent cell monolayers. The next day, the cells were fed fresh culture medium and culture was continued for at least 24 hours before analysis by flow cytometry.

Falcon 1008 (35-mm) bacteriological culture dishes were coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) at a concentration of 10 μg/cm^2 as described previously.[50] Murine BM cells were prestimulated for 2 days in their appropriate culture medium, as mentioned above, supplemented with 10 ng/mL human interleukin-6 (hIL-6; Ares-Serono, Geneva, Switzerland), 10 ng/mL murine IL-3 (mIL-3; Research & Development, Minneapolis, MN), and 100 ng/mL murine stem cell factor (mSCF; Immunex, Seattle, WA) at a starting cell concentration of 5×10^5 nucleated cells/mL. Before adding BM cells to the fibronectin-coated dishes, the CH-296 was preincubated with supernatant containing the ecotropic or amphotropic MFG-EGFP vector for 1 hour at 37°C.[51,52] Subsequently, nucleated cells were resuspended at 0.5 to 2.0 × 10^6/mL in vector-containing supernatant supplemented with hIL-6, mIL-3, and mSCF, and added to the dishes. Over a period of 2 days retrovirus supernatant was changed an additional four times, each time resuspending nonadherent cells into fresh retrovirus superna-



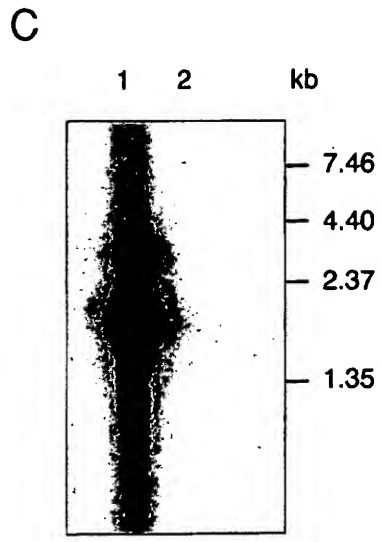


Fig 1. Characterization of the aMFG-EGFP retroviral producer cell line. (A) Schematic representation of the MFG-EGFP retroviral vector. LTR, long terminal repeat; ψ, viral packaging signal; SD and SA, splice donor and acceptor sites, respectively; *gag* and *env* indicate the presence of part of the MoMLV *gag* and *env* sequences. The predicted sizes of the two expected RNA isoforms are indicated. (B) FACS analysis of EGFP expression; the corresponding profile of the packaging cell line GP+*env*Am12 (thin line) is overlaid for comparison. (C) Northern blot analysis of aMFG-EGFP RNA. Total RNA (25 μg) from producer cells (lane 1) and GP+*env*Am12 (lane 2) was resolved by electrophoresis, blotted to a nylon membrane, and hybridized with a probe specific for EGFP.

tant. Mock transductions were standard and similarly performed with supernatants of the corresponding packaging cell lines GP+E-86 or GP+*env*Am12. Finally, the cells were procured and used for FACS analysis/sorting, and in vitro clonogenic progenitor, CFU-S, and MRA assays.

Flow cytometry. Cell samples were analyzed/sorted using a FACScan flow cytometer, a FACSCalibur, or a FACS Vantage cell sorter (Becton Dickinson, San Jose, CA). Green fluorescence was measured through a 530-nm/30-nm bandpass filter after illumination with the 488-nm line of an argon ion laser. Dead cells were excluded from analysis based on propidium iodine (Sigma) or Hoechst 33258 (Molecular Probes, Eugene, OR) staining. Immunophenotyping of EGFP- and mock-transduced BM cells was performed by staining with two rat monoclonal antibodies (MoAbs), ER-MP20 (Ly-6C) and ER-MP12, which recognize distinct subsets within the murine hematopoietic cell compartment.[53-55] Unlabeled ER-MP20 (hybridoma culture supernatant) and biotinylated ER-MP12 rat MoAbs were kindly provided by Dr M. de Bruijn (Department of Immunology, Erasmus University Rotterdam, The Netherlands). Cells were sequentially stained with unlabeled ER-MP20, Cyanine-5 conjugated goat-antirat antibodies (GaR-Cy5; Amersham, Buckinghamshire, UK), biotinylated ER-MP12, and Tricolor-conjugated streptavidin (SA-Tricolor; Caltag, San Francisco, CA). All incubations were performed in Hanks' Balanced Salt Solution, supplemented with 2% (wt/vol) bovine serum albumin, 0.05% (wt/vol) sodium azide, and 2% (vol/vol) heat-inactivated mouse serum. During the second incubation step, 2% (vol/vol) goat serum was added to block nonspecific binding, and before the third incubation, cells were incubated with 4% (vol/vol) rat serum to block residual binding sites on the GaR-Cy5 antibodies. Spillover of EGFP fluorescence in the FL3 channel and of Tricolor in the FL4 channel were electronically compensated using appropriately stained control samples.

In vitro clonogenic progenitor assays. Murine BM cells were plated at appropriate numbers in Falcon 1008 (35-mm) bacteriological culture dishes in 1 mL of semisolid methylcellulose culture medium containing 0.8% (wt/vol) methylcellulose (Methocel A4M Premium grade; Dow Chemical Co, Barendrecht, The Netherlands) in enriched DMEM, 1% (wt/vol) BSA, 0.3 mg/mL human transferrin, 0.1 μmol/L sodium selenite, 1 mg/L nucleosides (as above), 0.1 mmol/L β-mercaptoethanol, 15 μmol/L linoleic acid, 15 μmol/L cholesterol, 10 μg/mL insulin, 100 U/mL penicillin, and 100 μg/ml streptomycin.[42,56] Granulocyte/macrophage colony formation (GM-CFU) was stimulated by 10 ng/mL mIL-3, 100 ng/mL mSCF, and M-CSF purified from pregnant mouse uteri extract essentially as described before.[56] GM-CFUs were scored at day 8 to 10 of culture, individually isolated, and analyzed by FACS and/or PCR. BFU-E growth was induced by 100 ng/mL mSCF and 4 U/mL human erythropoietin (hEPO; Behringwerke, Marburg, Germany) in the presence of 2×10^{-4} mol/L hemin (bovine, type I; Sigma), whereas CFU-E growth was stimulated similarly with hEPO alone. CFU-E colonies were counted after 2 days and BFU-E colonies after 8 to 10 days of culture. Megakaryocyte progenitor cells (CFU-Meg) were stimulated in 0.375% agar cultures supplemented with 100 ng/mL mSCF, 10 ng/mL mIL-3, and 10 ng/mL murine thrombopoietin (mTPO; Genentech Inc, San Francisco, CA). Colonies were dried after 10 days, stained for acetylcholinesterase positive cells, and counted.[57] All cultures were grown in duplicate and maintained at 37°C with 10% CO_2/90% air in a humidified atmosphere.

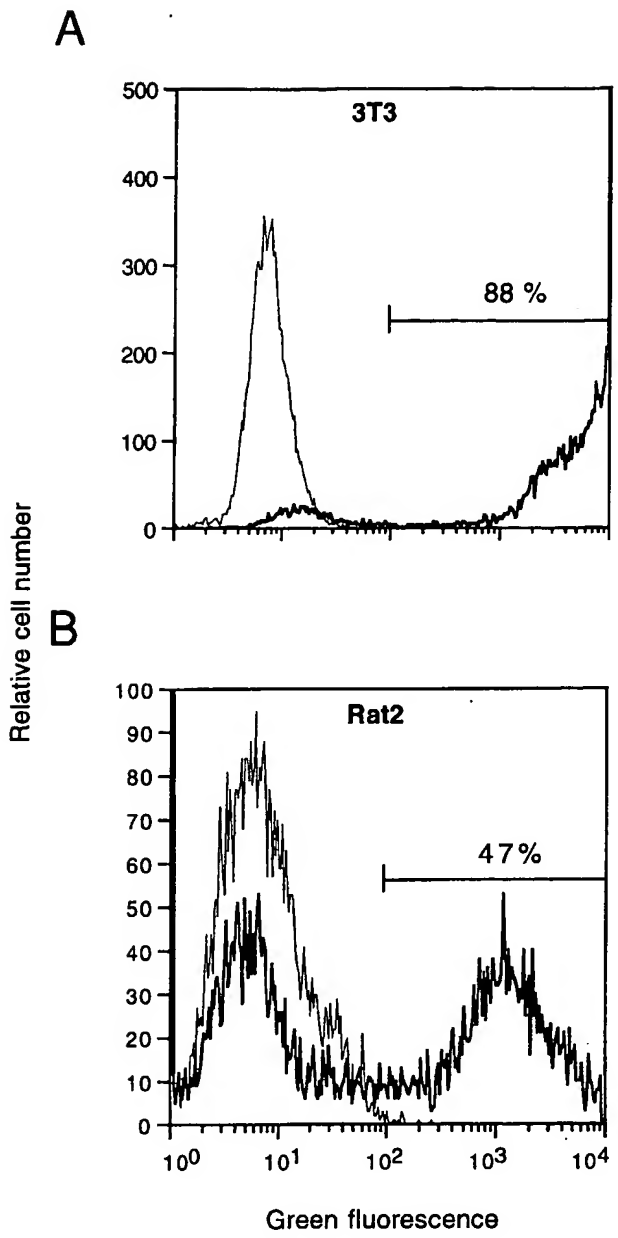


Fig 2. Flow cytometric analysis of EGFP expression in NIH 3T3 (A) and Rat2 (B) cells transduced with aMFG-EGFP retroviral-containing supernatant. The corresponding profiles for cells mock-transduced with GP+*env*Am12-derived supernatant (thin lines) are overlaid for comparison. Percentages of cells positive for EGFP expression are indicated.

CFU-S assay. Lethally irradiated recipient female BCBA mice (8.5 Gy, ^{137}Cs γ-rays) were injected intravenously with 0.75×10^4, 1.25×10^4, 1.5×10^4, or 2.5×10^4 eMFG-EGFP-transduced BM cells. As a control, irradiated mice were injected with BM cells treated similarly with supernatant of the ecotropic packaging cell line GP+E-86. Thirteen days later, mice were killed and total spleen and individual macroscopic spleen colonies were dissected and resuspended for flow cytometric analysis and in vitro clonogenic progenitor assays.

MRA assay. Lethally irradiated recipient female BCBA mice (8.5 Gy, ^{137}Cs γ-rays) were injected intravenously with 1×10^5 eMFG-EGFP-transduced BM cells or, as a control, with BM cells treated similarly with supernatant of the ecotropic packaging cell line GP+E-86. At day 13, the animals were killed and both the peripheral blood and femoral marrow content were analyzed by flow cytometry. The femoral marrow was assayed for the presence of in vitro clonogenic progenitors (GM-CFU) as mentioned above.

Amplification of genomic DNA sequences. Individually isolated colonies were disrupted by boiling and subsequently lysed for 1 hour at 56°C in *Taq* polymerase buffer (ie, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 10 mmol/L Tris-HCl, pH 9.0) supplemented with 0.1% gelatine, 0.5% NP40, 0.5% Tween 20, 4.3 μg/mL proteinase K, and 24 μg/mL RNAse A. After denaturation by boiling, genomic DNA sequences specific for EGFP and, as a positive control, the murine cannabinoid receptor (mCb2) were amplified by PCR.[58] The PCR reactions were performed with *Taq* polymerase buffer supplemented with 0.1% gelatine, 200 μmol/L dNTPs, 1 μmol/L primers, and 2.5 U of *Taq* polymerase under the following conditions: initial denaturation at 94°C for 5 minutes; followed by 40 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 58°C for mCb2 or at 64°C for EGFP) and polymerization (1 minute at 72°C). The primer sequences used to amplify a 709-bp fragment of the mCb2 gene were 5′-AGCGTGATCTTCGCCTGCAA-3′ and 5′-CTTCTTCCAGCCTATCAGGC-3′. Similarly, the primer sequences used to amplify a 468-bp fragment of the EGFP gene consisted of 5′-GAAGTTCATCTGCACCACCGGCAA-3′ and 5′-TAGTGGTTGTCGGGCAGCAGCACG-3′. The amplified products were purified by phenol/chloroform extraction and ethanol precipitation, resolved by 1% agarose gel electrophoresis, and analyzed by Southern blotting/hybridization using specific ^{32}P-labeled cDNA probes according to standard procedures.[59]

RESULTS

Generation of the MFG-EGFP retroviral producer cells. A retroviral vector was constructed in which the complete coding sequence of EGFP was placed under transcriptional control of the MoMLV LTR in the MFG retroviral backbone (Fig 1A). This MFG-EGFP plasmid was used to generate polyclonal cell populations of the ecotropic and amphotropic retroviral producer cell lines GP+E-86 and GP+*env*Am12, respectively, as described earlier.[41] Cells displaying the brightest green fluorescence were single-cell selected by FACS and expanded. Ecotropic (eMFG-EGFP) and amphotropic (aMFG-EGFP) clonal producer cell lines were thus established and characterized by flow cytometry and Northern blot analysis. As is shown in Fig 1B for aMFG-EGFP, over 95% of the viable cells displayed a bright green fluorescence upon excitation at 488 nm in the flow cytometer. Northern blot analysis of total RNA isolated from this cell line showed the presence of the predicted 1.9- and 2.8-kb RNA transcripts (Fig 1C). The eMFG-EGFP and aMFG-EGFP cell lines were producing recombinant retrovirus at titers of 10^5 and 10^6 infectious particles per milliliter, respectively, as assessed by EGFP gene transfer to NIH 3T3 cells. These retroviral producer cell lines have been cultured for at least 2 months without loss of this phenotype, indicating the stability and the absence of any detrimental effects of EGFP expression. The data show that selective cell sorting on the basis of EGFP expression can be applied for the isolation of stable and recombinant retrovirus producing cell clones.

To explore the potential of EGFP as a marker for monitor-

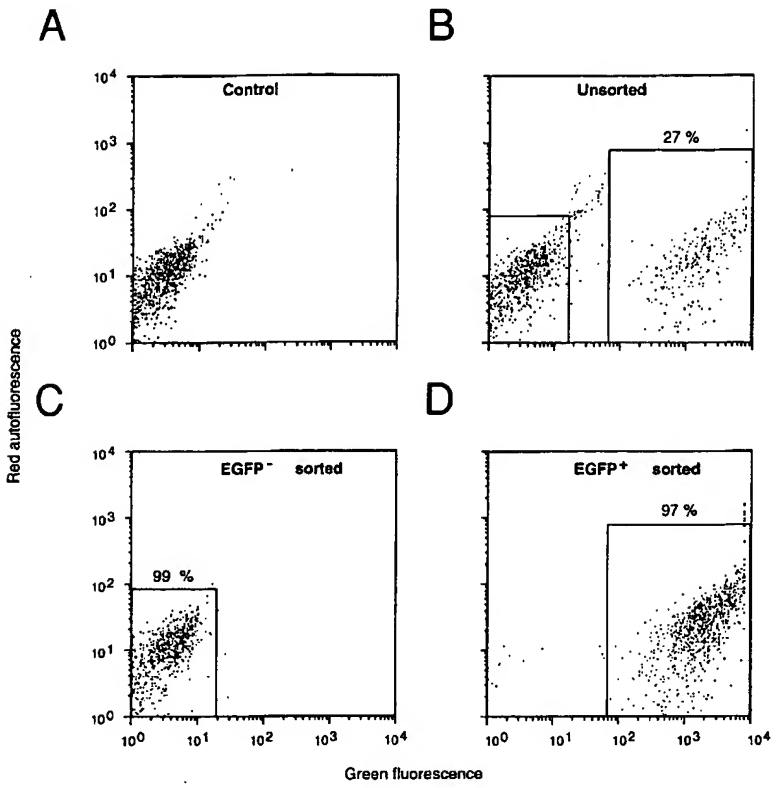


Fig 3. FACS selection of MFG-EGFP-transduced murine BM cells. Dot-plot profiles of mock-transduced (A), MFG-EGFP-transduced total BM cells (B), FACS sorted EGFP-negative (C), and EGFP-positive cells (D) are shown. The number of events obtained in the FL2 emission channel (= red autofluorescence) is plotted versus the number obtained in the FL1 channel (= green fluorescence). Percentages of cells in the sort windows are indicated. Cells from each of these fractions were used for in vitro clonogenic progenitor (GM-CFU) and day 13 CFU-S assays and individual colonies were analyzed for EGFP expression by FACS (Table 1).

ing gene transfer and expression in murine cells, we transduced both NIH 3T3 and Rat2 fibroblasts with supernatants from aMFG-EGFP. FACS analysis of viable transduced cells showed EGFP expression in 88% of the NIH 3T3 and 47% of the Rat2 cells under the conditions used (Fig 2), indicating the feasibility of detecting gene transfer efficiencies on the basis of EGFP expression.

Transduction and selection of primary murine BM cells. Normal murine BM cells were prestimulated with hIL-6, mIL-3, and mSCF for 2 days and subsequently transduced by a recombinant fibronectin-mediated supernatant transduction procedure in the presence of the same growth factors. Bacteriological culture dishes were coated with fibronectin fragment CH-296 and preloaded with retroviral vector before the addition of hematopoietic progenitor cells and fresh retroviral supernatant. Over a period of 2 days new retroviral supernatant was added four times. Subsequently, the cells were recovered from the dishes and analyzed directly by FACS. The transduction efficiencies obtained with this procedure ranged from 17% to 38% of the viable nucleated BM cells (four independent experiments). Sorting of the BM cells on the basis of EGFP expression showed that the transduced cells displayed a strong green fluorescent signal, well separated from the EGFP-negative cells (Fig 3B *v* Fig 3A). The BM cells could be FACS sorted into greater than 95% EGFP-negative and EGFP-positive cell populations (Fig 3C and D, respectively). Each cell fraction was evaluated for the presence of in vitro clonogenic progenitor cells in defined methylcellulose cultures; individual colonies were isolated and assayed for EGFP expression by flow cytometry (Table 1), fluorescence microscopy (Fig 4), or for proviral integration by PCR (Fig 5).

Table 1. EGFP Expression in Individual GM-CFU Methylcellulose Colonies and Day 13 CFU-S Derived From Mock, Unsorted, EGFP-Negative, and EGFP-Positive Sorted Murine BM Cells After Retroviral Transduction

Cells	No. EGFP-Positive GM-CFU/Total No. GM-CFU Analyzed (%)		No. EGFP-Positive CFU-S/Total No. CFU-S Analyzed* (%)	
Total mock	0/39	(0)	0/30	(0)
Total EGFP	29/73	(40)	38/50	(76)
EGFP-negative sorted	1/19	(5)	5/20	(25)
EGFP-positive sorted	24/25	(96)	17/20	(85)

Individual GM-CFU or day 13 CFU-S derived from the appropriate marrow fractions were isolated and analyzed for EGFP expression by flow cytometry as described in Materials and Methods. The cumulative results for four (GM-CFU) or two (CFU-S) independent experiments are shown.

* A CFU-S was determined to be EGFP-positive if >10% of the resuspended cells displayed a distinct green fluorescent signal.

EGFP-positive in vitro clonogenic progenitor cells could not be recovered from the total mock transduced BM cells, whereas only 1 of 19 GM-CFU recovered from the EGFP-negative sorted cell population proved to be EGFP-positive by FACS (Table 1). Approximately 40% of the in vitro clonogenic progenitors present in the total EGFP-transduced cells displayed EGFP expression (cumulative results of four independent experiments), whereas greater than 95% of those from the EGFP-positive sorted cells proved to be posi-

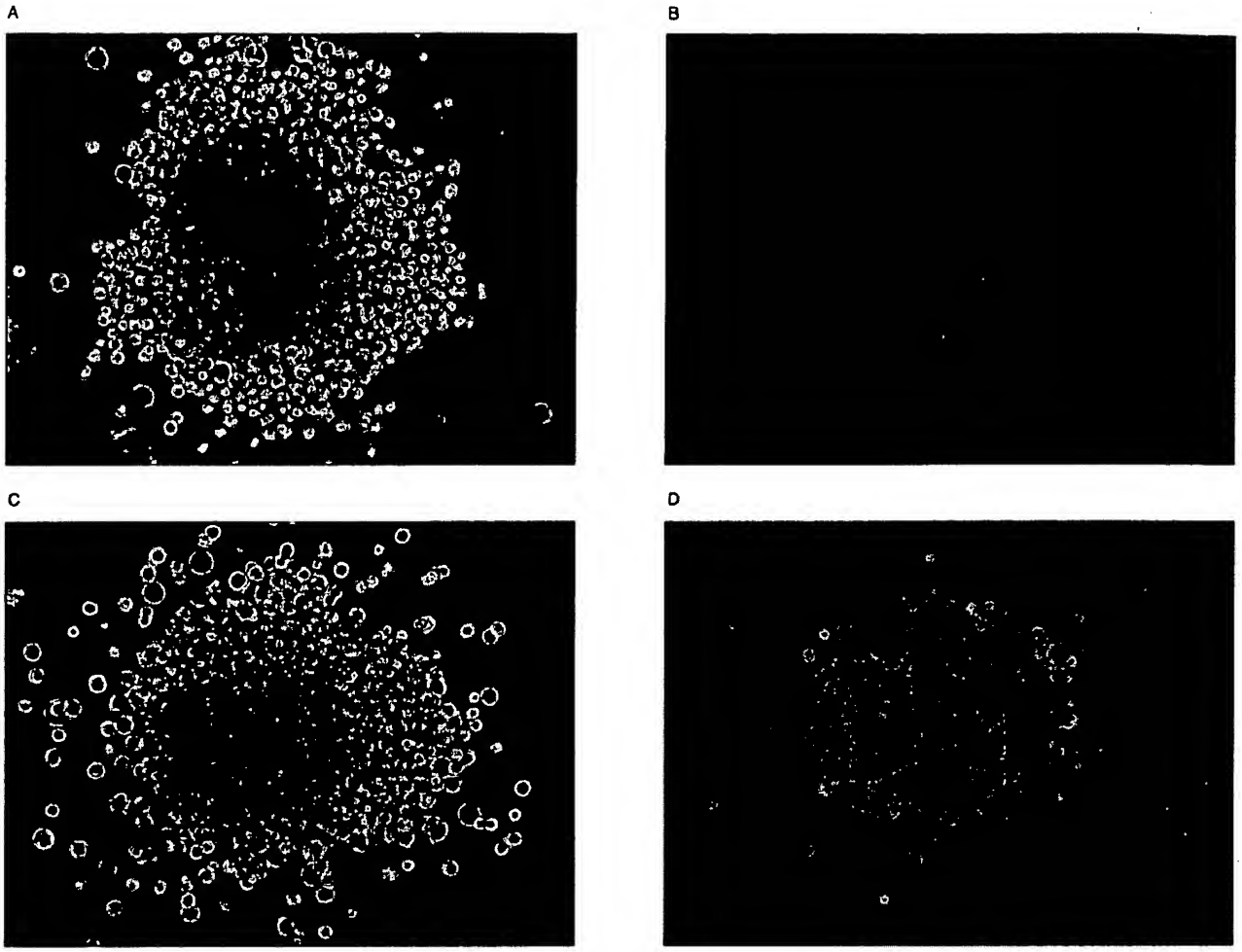


Fig 4. Examination of EGFP expression in individual GM-CFU methylcellulose colonies by inverted fluorescence microscopy. Murine BM cells were transduced with the MFG-EGFP retroviral vector and subsequently analyzed for the presence of in vitro clonogenic progenitor cells. Individual GM-CFU were then examined with an Olympus IX70 inverted fluorescence microscope. Phase-contrast (A, C) and fluorescence micrographs (B, D) were prepared of an EGFP-negative (A, B) and an EGFP-positive GM-CFU (C, D); original magnification × 100.

tive by flow cytometric analysis. For further illustration of EGFP expression in in vitro clonogenic progenitor cells, fluorescence micrographs of an EGFP-negative and an EGFP-positive GM-CFU are shown in Fig 4. Similar results were obtained when individual colonies derived from the different fractions were analyzed for the presence of the provirus (Fig 5). The almost exclusive presence of the MFG-EGFP provirus in the EGFP-positive sorted in vitro clonogenic progenitors is most likely the result of the relatively high fluorescence intensity differences between the EGFP-negative and EGFP-positive cells and the stringent sorting windows chosen. These results show that it is possible to enrich for retrovirally transduced in vitro clonogenic progenitors on the basis of EGFP expression. Moreover, EGFP expression remained stable without any selective pressure during methylcellulose cultures.

EGFP expression in CFU-S and marrow-repopulating cells. To evaluate the presence and expression of the MFG-EGFP retroviral vector in CFU-S and marrow repopulating cells, murine BM cells were transduced with the eMFG-EGFP retroviral vector and injected into lethally irradiated recipients. For analysis of EGFP expression, individual spleen colonies were dissected at day 13 posttransplantation and analyzed by flow cytometry. Examples of the fluorescence profiles of EGFP-negative and EGFP-positive CFU-S colonies are shown in Fig 6A and B, respectively. Whereas none of the 30 individual spleen colonies analyzed for the mock transduction provided for a positive green fluorescence signal, 38 of the 50 spleen colonies (76%) derived from mice injected with total MFG-EGFP–transduced BM cells displayed EGFP expression in over 10% of the resuspended cells (Table 1). This number of EGFP-positive colonies increased to 85% (17/20) when the CFU-S were derived from mice injected with EGFP-positive selected BM cells. In contrast, only 5 of 20 CFU-S (25%) proved to be positive when EGFP-negative sorted BM cells were used as the donor cells.

An individual control, EGFP-negative and EGFP-positive CFU-S colony were assayed for EGFP expression in secondary GM-CFU. As determined by FACS analysis, EGFP-positive secondary in vitro clonogenic progenitor cells could

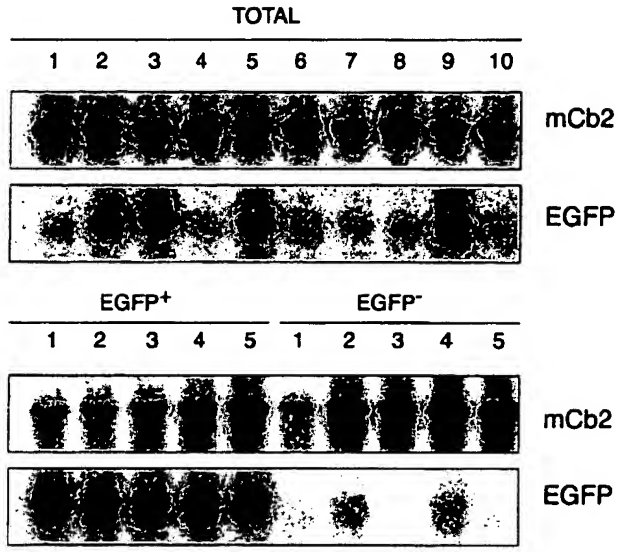


Fig 5. Demonstration of proviral integration analysis of in vitro clonogenic progenitor cells derived from retrovirally transduced murine BM cells. Individual colonies were isolated from methylcellulose cultures containing total EGFP-transduced BM cells, FACS-sorted EGFP-negative or EGFP-positive cells, and analyzed by PCR using primers specific for EGFP (EGFP). As a positive control for PCR, primers specific for the murine peripheral cannabinoid receptor (mCb2) were used.[58]

only be detected in the EGFP-positive spleen colony, but not in the control or EGFP-negative spleen colony. Examples of an EGFP-negative and EGFP-positive GM-CFU are shown in Fig 6C and Fig D, respectively. These results indicate that EGFP expression remained stable during the in vivo generation of CFU-S and the following secondary in vitro clonogenic progenitor assays.

The influence of EGFP expression on the outgrowth and differentiation potential of immature hematopoietic cells, present in the dissected spleen colonies, was evaluated by enumeration of progenitor cells along the granulocyte/macrophage, erythroid, and megakaryocyte lineages. As shown in Fig 7, individual spleen colonies which expressed EGFP in greater than 10% of the cells, or more stringently, in greater than 50% of the cells, contained 10^4 to 10^5 progenitor cells which was similar to the number obtained with mock-transduced or primary BM derived spleen colonies. The distribution of progenitor cells in individual spleen colonies was also not significantly different (Fisher's exact test) in the four groups examined. These data show that expression of EGFP does not impair multilineage outgrowth of immature hematopoietic cells.

Total spleen, BM, and peripheral blood of lethally irradiated recipient BCBA mice were also studied on day 13 post-transplantation for the presence of EGFP expression. As is shown in Fig 8 for one representative animal, FACS analysis revealed EGFP expression in 45% of the spleen cells (Fig 8A), 27% of femoral BM cells (Fig 8B), and 43% of periph-

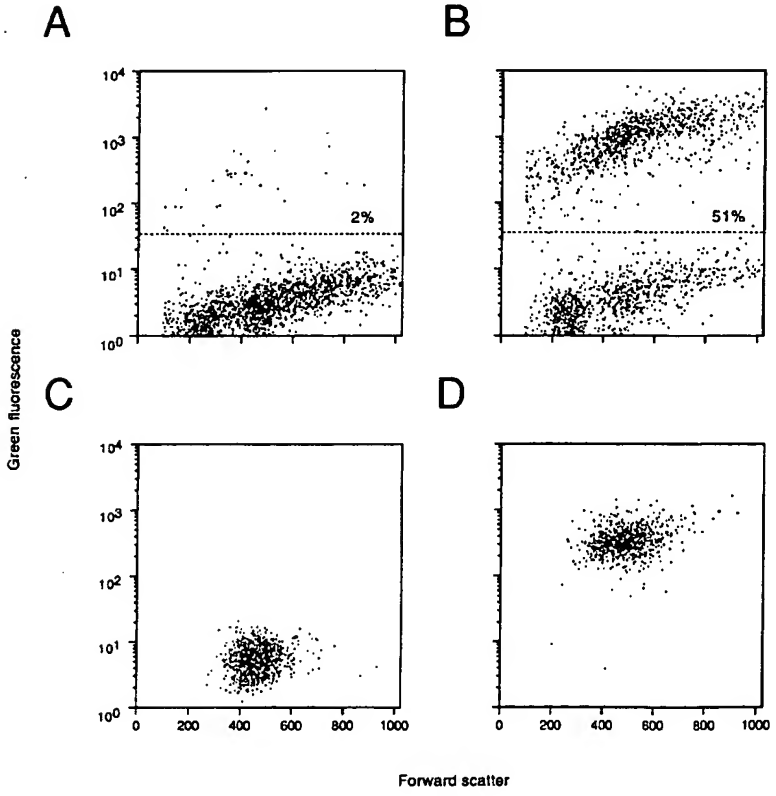


Fig 6. Demonstration of EGFP expression in individual isolated spleen colonies and GM-CFU derived thereof. Mice were injected with MFG-EGFP-transduced murine BM cells and 13 days later individual spleen colonies were dissected. The viable cells were analyzed by flow cytometry and cultured in standard colony assays for the presence of in vitro clonogenic progenitor cells. Examples are shown of an EGFP-negative (A) and EGFP-positive (B) spleen colony as well as a representative GM-CFU derived from these colonies (C and D, respectively). Percentages of cells positive for EGFP expression are indicated.

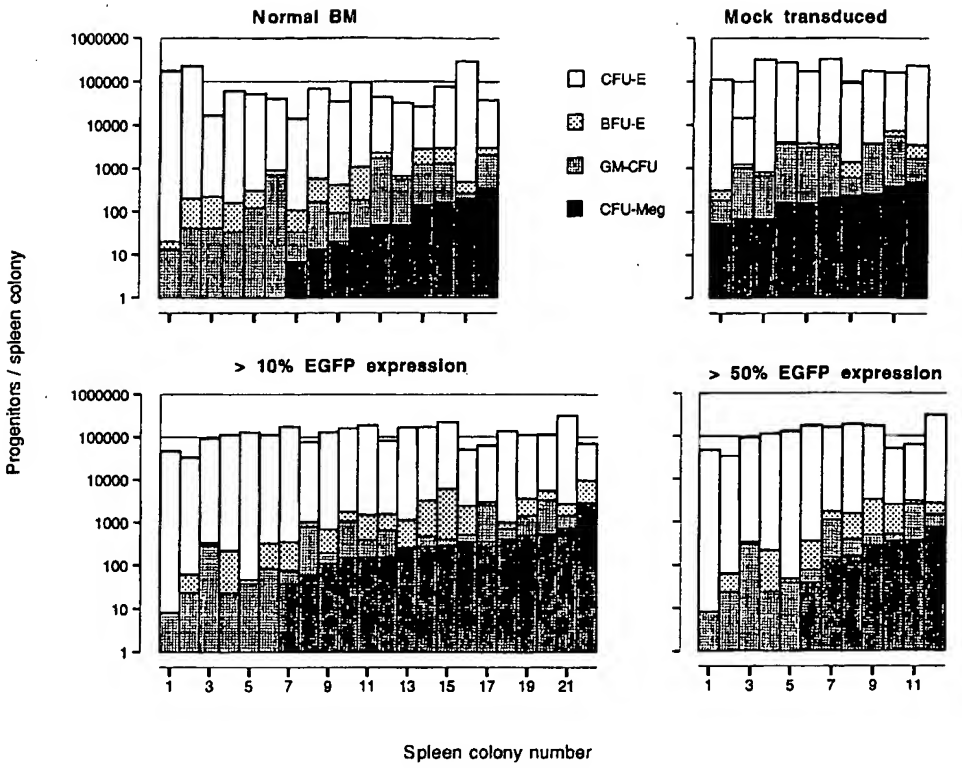


Fig 7. Distribution of erythroid, granulocyte/macrophage, and megakaryocytic progenitor cells in individual dissected spleen colonies derived from primary normal BM cells, total mock-transduced, and total MFG-EGFP–transduced BM cells. For the mock transduction of BM cells supernatant derived from the corresponding packaging cell line was used.

eral blood cells (Fig 8C), whereas the corresponding cells derived from control animals were negative. A bright green fluorescent signal was detected in 30% to 55% of the peripheral blood nucleated cells of 6 out of 6 animals studied in this way. Moreover, EGFP expression was also observed in secondary GM-CFU derived from the BM in contrast to those isolated similarly from control BM (results not shown). Taken together, these results show that EGFP is expressed in readily detectable frequencies in day 13 CFU-S, circulating blood cells, BM cells, and in secondary clonogenic progenitors recovered from the CFU-S colonies and BM.

Immunophenotyping of retrovirally transduced BM cells. To examine the transduction efficiency in different hematopoietic subsets, EGFP expression in transduced BM cells was compared with expression of the ER-MP12 and ER-MP20 antigens. Differential expression of these antigens has been shown to identify distinct immature hematopoietic cell populations in murine BM.[54,55] For example, the subpopulation of cells that is defined as ER-MP12-positive and ER-MP20-negative appeared to contain cells with long-term repopulating, day 12 spleen colony-forming ability, and clonogenic progenitors, whereas ER-MP20-positive and ER-MP12-negative cells consist of mature granulocytes.[55] Using antibodies specific for these antigens in three-color flow cytometric analysis the total EGFP-transduced cell population could be divided into four subsets (Fig 9A). EGFP expression was detected in approximately 34% of the ER-MP12-positive cells and in only 13% of the ER-MP12-negative cells (Fig 9B). Similarly, approximately 40% of the ER-MP20-negative cells and only 5% of the ER-MP20-positive cells displayed a bright green fluorescence (Fig 9C). These results indicate that the cell fraction described to contain immature hematopoietic cells is preferentially transduced with the MFG-EGFP retroviral vector, whereas the fraction that contains relatively mature myeloid cells contains few transduced cells. Moreover, these data show the feasibility of using EGFP expression as a convenient marker to determine transduction efficiencies in phenotypically defined hematopoietic cell populations.

DISCUSSION

In this study we investigated the potential use of EGFP as a retrovirally encoded reporter molecule in the genetic modification and selection of murine BM cells. Since long-term expression of recombinant proteins from the MFG retroviral vector has been shown in lethally irradiated recipient mice after reconstitution of the hematopoietic system with retrovirally transduced murine BM cells,[48,60] the EGFP gene was placed under transcriptional control of the MoMLV LTR in this vector. Both clonal ecotropic and amphotropic virus producer cell lines were generated using single-cell FACS cloning followed by cell expansion, and subsequently characterized. Flow cytometric analysis of these cells showed a bright green fluorescence in over 95% of the cells whereas Northern analysis confirmed the presence of the predicted vector-specific RNA species, indicating correct processing and splicing of the RNA and expression of the protein. Interestingly, the level of spliced RNA appears to be several-fold higher than that of unspliced RNA, a feature that has recently been correlated with augmented expression from the MFG retroviral vector.[60] Despite earlier reports on the instability of retroviral producers containing GFP-specific vector se-

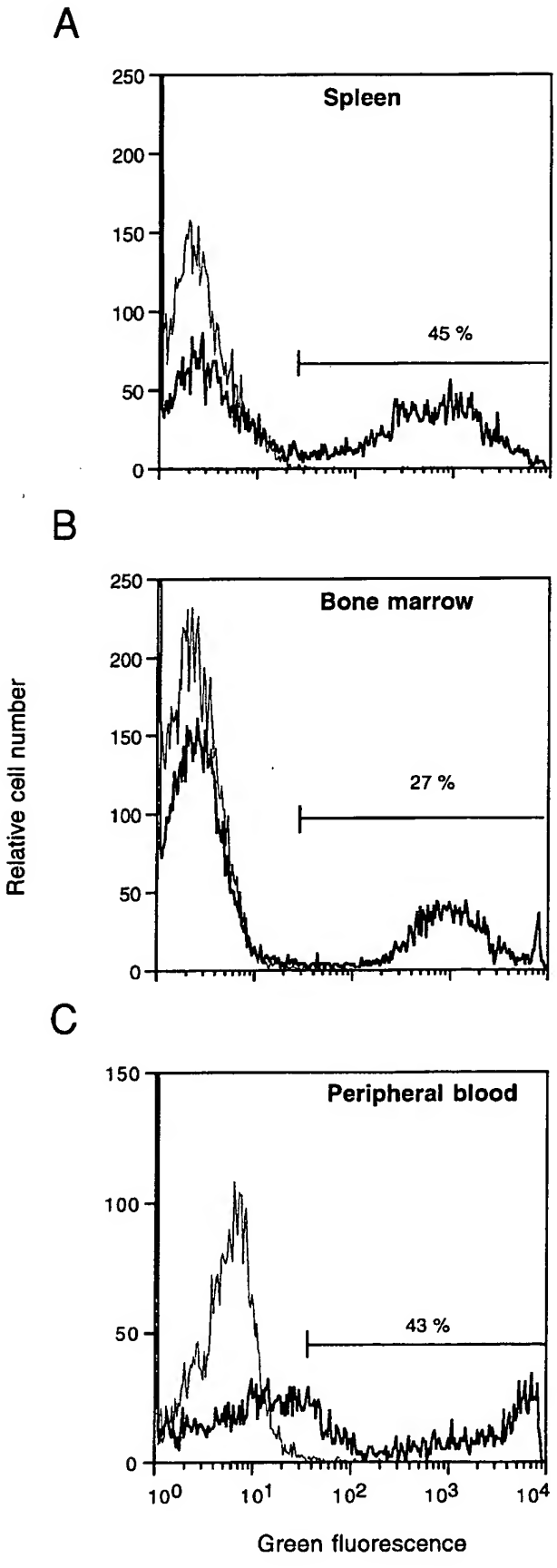


Fig 8. Flow cytometric analysis of EGFP expression 13 days post-transplantation in the spleen (A), BM (B), and peripheral blood (C) of mice injected with mock-transduced (thin lines) or MFG-EGFP–transduced murine BM cells. The results for one representative animal are shown. Percentages of cells positive for EGFP expression are indicated.

quences,[61,62] the eMFG-EGFP and aMFG-EGFP producer cell lines described here proved to be stable with respect to EGFP expression and high-titer virus production.

The utility of EGFP as a reporter molecule of retroviral-mediated gene transfer was then tested in murine cell lines and primary hematopoietic cells. Flow cytometric analysis together with functional studies showed EGFP expression directly after termination of the transduction procedure in NIH 3T3 and Rat2 cell lines, as well as in primary BM in vitro clonogenic progenitors and day 13 CFU-S. Moreover, EGFP expression was readily detectable by FACS in the peripheral blood of lethally irradiated recipient mice 13 days posttransplantation with retrovirally transduced BM cells as well as in secondary BM in vitro clonogenic progenitors. The latter marrow repopulating assay demonstrated stable EGFP expression in actively proliferating primary hematopoietic cells during approximately 1 month after transduction. FACS-determined transduction efficiencies in primary BM cells correlated well with the percentage positive in vitro clonogenic progenitors as determined by flow cytometric and proviral integration analysis of individual methylcellulose colonies. The corresponding transduction frequencies shown by FACS and PCR analysis for the in vitro clonogenic progenitors suggest that EGFP expression is, at least directly after transduction, optimal from the MFG vector in these cells. Similarly, the recovery of EGFP-positive day 13 CFU-S and marrow repopulating cells indicates directly that the MoMLV promotor is able to provide for high-level EGFP expression in these immature hematopoietic cell populations of BM. However, a clear discrepancy was noticed in the transduction efficiency of primary BM cells and the CFU-S derived thereof (17% to 38% *v* 76%). A likely explanation for this phenomenon might be that the combination of growth factors and the use of a medium that promotes the in vitro proliferation of CFU-S[43] for the prestimulation and transduction of murine BM cells has resulted in preferential transduction of immature cells that generate the macroscopic spleen colonies studied here. In this respect it is interesting that immunophenotyping of total EGFP-transduced murine BM cells with antibodies directed against the ER-MP12 and ER-MP20 antigens showed a preferential transduction of an ER-MP12-positive/ER-MP20-negative cell population, which recently has been correlated with the enriched presence of cells with long-term repopulating and spleen colony-forming ability.[55] The availability of the EGFP retroviral marker together with two- or possibly three-color flow cytometric analysis will further facilitate the identification of transduction conditions for phenotypically defined hematopoietic cells capable of long-term reconstitution. It should be noted that day 13 CFU-S are cells associated with short-term (ie, several months) reconstitution of irradiated recipients, while

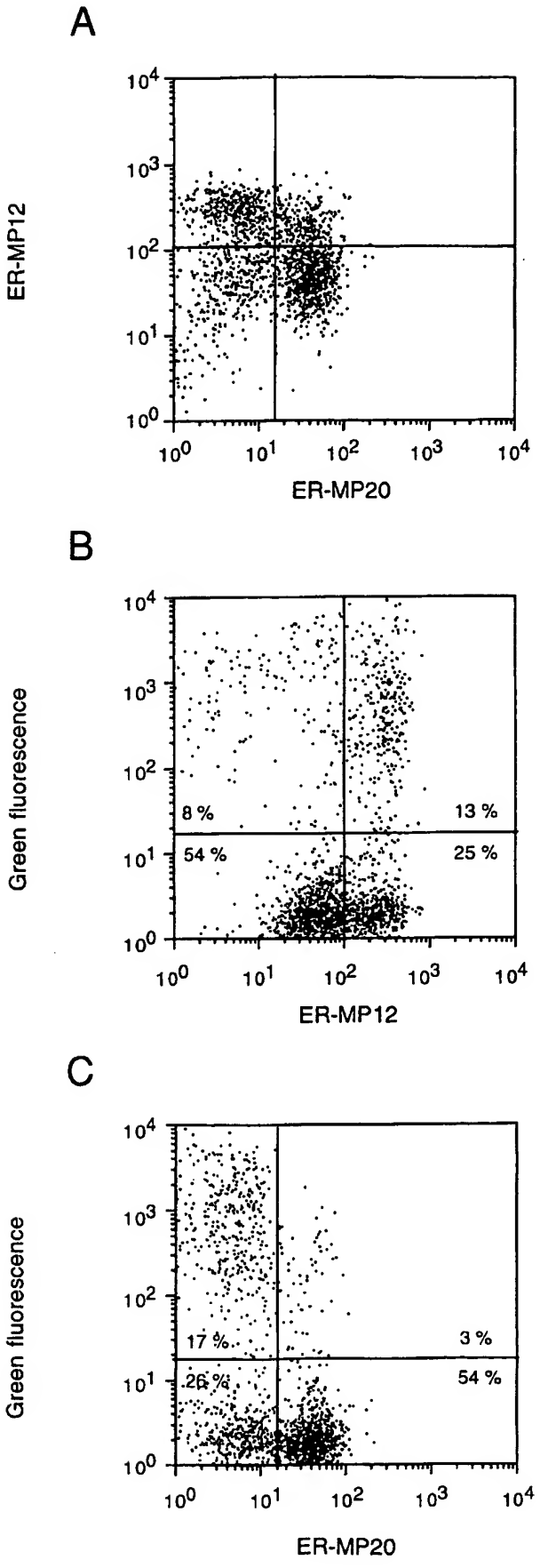


Fig 9. FACS analysis of EGFP expression in relation to ER-MP12 and ER-MP20 antigen expression in normal murine BM cells directly after transduction. The cells were stained sequentially with unlabeled ER-MP20 followed by GaR-Cy5 for detection of ER-MP20 antigen expression in the FL4 channel (ER-MP20), and with biotinylated ER-MP12 followed by SA-Tricolor for detection of ER-MP12 antigen expression in the FL3 channel (ER-MP12) of the FACSCalibur. The dot-plot profiles of ER-MP12 versus ER-MP20 (A) and of ER-MP12 and ER-MP20 versus green fluorescence (B and C, respectively) are shown. The lines used to discriminate between negative and positive cell populations are indicated and defined on the basis of appropriate control samples. In (B) and (C) the percentages of cells occurring in each quadrant are indicated.

cells with marrow repopulating ability correlate to long-term repopulating cells.[63] In this stage it remains to be determined whether cells capable of long-term hematopoietic reconstitution have been transduced as well and whether EGFP expression is still detectable in defined hematopoietic cell types at 6 months posttransplantation.

The feasibility of using EGFP as a marker for the selection of retrovirally transduced target cells was evaluated for BM in vitro clonogenic progenitor cells and day 13 spleen colonies. Retrovirally transduced BM cells were sorted on the basis of green fluorescence intensity in greater than 95% EGFP-negative and EGFP-positive cell populations and these fractions were used for in vitro clonogenic progenitor assays. Flow cytometry showed that ≥90% of the GM-CFU derived from the EGFP-positive population proved to be EGFP-positive; in contrast, ≥90% of the GM-CFU derived from the EGFP-negative population proved to be EGFP-negative. The percentage EGFP-negative and EGFP-positive day 13 CFU-S obtained from mice injected with the corresponding FACS sorted BM populations, however, were less stringent (25% and 85%, respectively). The generation of EGFP-positive CFU-S from negatively selected primary BM cells might be due to the lack of an extensive expression period in between the end of the transduction and the FACS sorting in our procedure. Differences between EGFP-negative and EGFP-positive primary BM cells were not observed with respect to the number and morphology of the derived GM-CFU (see, for example, Fig 4) and day 13 CFU-S (data not shown), and to the multilineage production of secondary progenitor cells in individual spleen colonies. These results clearly show the feasibility of using EGFP as a rapid selectable marker of retroviral-mediated gene transfer in primary hematopoietic cells.

The application of EGFP as a retrovirally encoded reporter for murine BM cells might be advantageous above the use of other selectable markers such as, eg, the bacterial β-galactosidase gene[22] and the human CD24 cell-surface antigen.[28] EGFP has no requirement for secondary molecules, such as antibodies or substrates, for analysis of expression by FACS or fluorescence microscopy and therefore limits time and effort necessary for the detection of the transduced cells. The fluorescence intensity differences between EGFP-positive and EGFP-negative murine BM cells are relatively high, thus facilitating and improving the efficiency of the FACS selection of retrovirally transduced cells. As with, eg,

the human CD24 cell-surface antigen, EGFP expression can be directly related to additional phenotypic markers (as demonstrated here for the cell-surface ER-MP12 and ER-MP20 antigens) using multiparameter flow cytometry to test, eg, various retroviral infection protocols for therapeutically relevant hematopoietic cells and to isolate pure populations of transduced cells with defined phenotypes, which will allow for functional analysis of their intrinsic properties. The use of EGFP as a reporter molecule might further facilitate studies on features of retroviral vector design, such as transcriptional control sequences, and serve as a fusion partner for biologically and/or therapeutically relevant genes. This marker might also facilitate in vivo studies on the homing and (growth-factor–stimulated) proliferation/differentiation of transplanted stem cells and the distribution properties of their progeny.

ACKNOWLEDGMENT

We are grateful to Dr R.C. Mulligan (Whitehead Institute for Biomedical Research, Cambridge, MA) for providing the MFG-lacZ plasmid and to Dr M.F.T.R. de Bruijn (Erasmus University Rotterdam, The Netherlands) for the ER-MP12 and ER-MP20 antibodies; Genetix Pharmaceuticals (Tarrytown, New York) for providing the GP+E-86 and GP+*env*Am12 packaging cell lines; Takara Shuzo Co (Shiga, Japan) for supplying CH-296; Ares-Serono (Geneva, Switzerland) for providing the hIL-6; Immunex (Seattle, WA) for providing the mSCF; Behringwerke (Marburg, Germany) for providing the hEPO; and Genentech Inc (San Francisco, CA) for providing the mTPO and mIL-3. We gratefully acknowledge K. van Rooyen for photographical artwork and E. van Bodegom for taking care of the mice.

REFERENCES

1. Nienhuis AW, McDonagh KT, Bodine DM: Gene transfer into hematopoietic stem cells. Cancer 67:2700, 1991

2. Einerhand MPW, Valerio D: Gene transfer into hematopoietic stem cells: Prospects for human gene therapy. Curr Top Microbiol Immunol 177:217, 1992

3. Kerr WG, Mulé JJ: Gene therapy: Current status and future prospects. J Leukoc Biol 56:210, 1994

4. Vile RG, Russell SJ: Retroviruses as vectors. Br Med Bull 51:12, 1995

5. Brenner MK, Cunningham JM, Sorrentino BP, Heslop HE: Gene transfer into human hemopoietic progenitor cells. Br Med Bull 51:167, 1995

6. Dunbar CE, Emmons RVB: Gene transfer into hematopoietic progenitor and stem cells: Progress and problems. Stem Cells 12:563, 1994

7. Miller DG, Adam MA, Miller AD: Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 10:4239, 1990

8. Springett GM, Moen RC, Anderson S, Blaese RM, Anderson WF: Infection efficiency of T lymphocytes with amphotropic retroviral vectors is cell cycle dependent. J Virol 63:3865, 1989

9. Stead RB, Kwok WW, Storb R, Miller AD: Canine model for gene therapy: Inefficient gene expression in dogs reconstituted with autologous marrow infected with retroviral vectors. Blood 71:742, 1988

10. Challita P-M, Kohn DB: Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation *in vivo*. Proc Natl Acad Sci USA 91:2567, 1994

11. Ogawa M: Differentiation and proliferation of hematopoietic stem cells. Blood 81:2844, 1993

12. Lemischka IR: The haematopoietic stem cell and its clonal progeny: Mechanisms regulating the hierarchy of primitive haematopoietic cells. Cancer Surveys 15:3, 1992

13. Szilvassy SJ, Cory S: Efficient retroviral gene transfer to purified long-term repopulating hematopoietic stem cells. Blood 84:74, 1994

14. Williams DA, Lemischka IR, Nathan DG, Mulligan RC: Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. Nature 310:476, 1984

15. Jordan CT, Lemischka IR: Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Dev 4:220, 1990

16. Van Beusechem VW, Kukler A, Heidt PJ, Valerio D: Long-term expression of human adenosine deaminase in rhesus monkeys transplanted with retrovirus-infected bone-marrow cells. Proc Natl Acad Sci USA 89:7640, 1992

17. Carter RF, Abrams-Ogg CG, Dick JE, Kruth SA, Valli VE, Kamel-Reid S, Dubé ID: Autologous transplantation of canine long-term marrow culture cells genetically marked by retroviral vectors. Blood 79:356, 1992

18. Dunbar CE, Cottler-Fox M, O'Shaughnessy JA, Doren S, Carter C, Berenson R, Brown S, Moen RC, Greenblatt J, Stewart FM, Leitman SF, Wilson WH, Cowan K, Young NS, Nienhuis AW: Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. Blood 85:3048, 1995

19. Brenner MK, Rill DR, Holladay MS, Heslop HE, Moen RC, Buschle M, Krance RA, Santana VM, Anderson WF, Ihle JN: Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. Lancet 342:1134, 1993

20. Brenner MK, Rill DR, Moen RC, Krance RA, Mirro J Jr, Anderson WF, Ihle JN: Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. Lancet 341:85, 1993

21. Brenner MK: The contribution of marker gene studies to hemopoietic stem cell therapies. Stem Cells 13:453, 1995

22. Strair RK, Towle M, Smith BR: Retroviral mediated gene transfer into bone marrow progenitor cells: Use of beta-galactosidase as a selectable marker. Nucleic Acids Res 18:4759, 1990

23. Nolan GP, Fiering S, Nicolas J-F, Herzenberg LA: Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli lacZ*. Proc Natl Acad Sci USA 85:2603, 1988

24. Staal FJT, Bakker AQ, Verkuijlen M, Van Oort E, Spits H: Use of bicistronic retroviral vectors encoding the LacZ gene together with a gene of interest: A method to select producer cells and follow transduced target cells. Cancer Gene Ther 3:345, 1996

25. Staal FJT, Res PCM, Weijer K, Spits H: Development of retrovirally marked human T progenitor cells into mature thymocytes. Int Immunol 7:1301, 1995

26. Ward M, Richardson C, Pioli P, Smith L, Podda S, Goff S, Hesdorffer C, Bank A: Transfer and expression of the human multiple drug resistance gene in human CD34$^+$ cells. Blood 84:1408, 1994

27. Conneally E, Bardy P, Eaves CJ, Thomas T, Chappel S, Shpall EJ, Humphries RK: Rapid and efficient selection of human hematopoietic cells expressing murine heat-stable antigen as an indicator of retroviral-mediated gene transfer. Blood 87:456, 1996

28. Pawliuk R, Kay R, Lansdorp P, Humphries RK: Selection of retrovirally transduced hematopoietic cells using CD24 as a marker of gene transfer. Blood 84:2868, 1994

29. Mavilio F, Ferrari G, Rossini S, Nobili N, Bonini C, Casorati G, Traversari C, Bordignon C: Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer. Blood 83:1988, 1994

30. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC: Green fluorescent protein as a marker for gene expression. Science 263:802, 1994

31. Prasher DC, Eckenrode VK, Ward WW, Prendergast FG,

Cormier MJ: Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111:229, 1992

32. Morin JG, Hastings JW: Energy transfer in a bioluminescent system. J Cell Physiol 77:313, 1971

33. Ward WW, Cody CW, Hart RC, Cormier MJ: Spectrophotometric identity of the energy-transfer chromophores in *Renilla* and *Aequorea* green fluorescent proteins. Photochem Photobiol 31:611, 1980

34. Delagrave S, Hawtin RE, Silva CM, Yang MM, Youvan DC: Red-shifted excitation mutants of the green fluorescent protein. Bio-Technology 13:151, 1995

35. Heim R, Tsien RY: Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr Biol 6:178, 1996

36. Zhang G, Gurtu V, Kain SR: An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. Biochem Biophys Res Commun 227:707, 1996

37. Cormack BP, Valdivia RH, Falkow S: FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:33, 1996

38. Cheng L, Fu J, Tsukamoto A, Hawley RG: Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. Nature Biotechnol 14:606, 1996

39. Levy JP, Muldoon RR, Zolotukhin S, Link CJ Jr: Retroviral transfer and expression of a humanized, red-shifted green fluorescent protein gene into human tumor cells. Nature Biotechnol 14:610, 1996

40. Lybarger L, Dempsey D, Franek KJ, Chervenak R: Rapid generation and flow cytometric analysis of stable GFP-expressing cells. Cytometry 25:211, 1996

41. Bierhuizen MFA, Westerman Y, Visser TP, Wognum AW, Wagemaker G: Green fluorescent protein variants as markers of retroviral-mediated gene transfer in primary hematopoietic cells and cell lines. Biochem Biophys Res Commun 234:371, 1997

42. Wagemaker G, Visser TP: Erythropoietin-independent regeneration of erythroid progenitor cells following multiple injections of hydroxyurea. Cell Tissue Kinet 13:505, 1980

43. Wagemaker G: Hematopoietic cell differentiation, in Van Bekkum DW, Löwenberg B (eds): Bone Marrow Transplantation. New York, NY, Dekker, 1985, p 1

44. Wagemaker G: Selective multiplication of repopulating hemopoietic stem cells in vitro for bone marrow transplantation. Transplant Proc 19:2721, 1987

45. Markowitz D, Goff S, Bank A: A safe packaging cell line for gene transfer: Separating viral genes on two different plasmids. J Virol 62:1120, 1988

46. Markowitz D, Goff S, Bank A: Construction and use of a safe and efficient amphotropic packaging cell line. Virology 167:400, 1988

47. Topp WC: Normal rat cell lines deficient in nuclear thymidine kinase. Virology 113:408, 1981

48. Rivière I, Brose K, Mulligan RC: Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. Proc Natl Acad Sci USA 92:6733, 1995

49. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156, 1987

50. Moritz T, Patel VP, Williams DA: Bone marrow extracellular matrix molecules improve gene transfer into human hematopoietic cells via retroviral vectors. J Clin Invest 93:1451, 1994

51. Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA: Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nature Med 2:876, 1996

52. Moritz T, Dutt P, Xiao X, Carstanjen D, Vik T, Hanenberg H, Williams DA: Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: Evidence of direct viral binding to chymotryptic carboxy-terminal fragments. Blood 88:855, 1996

53. Leenen PJM, Melis M, Slieker WAT, Van Ewijk W: Murine macrophage precursor characterization. II. Monoclonal antibodies against macrophage precursor antigens. Eur J Immunol 20:27, 1990

54. De Bruijn MFTR, Slieker WAT, Van der Loo JCM, Voerman JSA, Van Ewijk W, Leenen PJM: Distinct mouse bone marrow macrophage precursors identified by differential expression of ER-MP12 and ER-MP20 antigens. Eur J Immunol 24:2279, 1994

55. Van der Loo JCM, Slieker WAT, Kieboom D, Ploemacher RE: Identification of hematopoietic stem cell subsets on the basis of their primitiveness using antibody ER-MP12. Blood 85:952, 1995

56. Merchav S, Wagemaker G: Detection of murine bone marrow granulocyte/macrophage progenitor cells (GM-CFU) in serum free cultures stimulated with purified M-CSF or GM-CSF. Int J Cell Cloning 2:356, 1984

57. Bauman JGJ, Chen MG: Light scatter and cell surface properties of murine megakaryocyte progenitor cells. Exp Hematol 15:1074, 1987

58. Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Pessègue B, Bonnin-Cabanne O, Le Fur G, Caput D, Ferrara P: Molecular cloning, expression and function of the murine CB2 peripheral cannabinoid receptor. Biochim Biophys Acta 1307:132, 1996

59. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1989

60. Krall WJ, Skelton DC, Yu X-J, Rivière I, Lehn P, Mulligan RC, Kohn DB: Increased levels of spliced RNA account for augmented expression from the MFG retroviral vector in hematopoietic cells. Gene Ther 3:37, 1996

61. Hanazono Y, Dunbar CE, Emmons RVB: Retroviral producer cell lines containing green fluorescent protein S65T vector sequences are unstable: Evidence for toxicity in mammalian cells. Exp Hematol 24:1058, 1996 (abstr)

62. Hanazono Y, Dunbar CE, Emmons RVB: Low titer and high recombination frequency in green fluorescent protein retroviral vector producer cells suggest a selective disadvantage. Blood 88:434a, 1996 (abstr, suppl 1)

63. Van der Loo JCM, Van den Bos C, Baert MRM, Wagemaker G, Ploemacher RE: Stable multilineage hematopoietic chimerism in α-thalassemic mice induced by a bone marrow subpopulation that excludes the majority of day-12 spleen colony-forming units. Blood 83:1769, 1994

NOTE *Physiology*

Identification of Skeletal Muscle Satellite Cells by Transfecting EGFP Driven by Skeletal α-Actin Promoter

Keitaro YAMANOUCHI[1], Chie SOETA[1]*, Shunichi SUZUKI[1], Telhisa HASEGAWA[2], Kunihiko NAITO[1] and Hideaki TOJO[1]

[1]*Laboratory of Applied Genetics, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657 and* [2]*Laboratory of Molecular and Cellular Biology, Japan Racing Association, 321-4 Tokami-cho, Utsunomiya, Tochigi 320-0856, Japan*

(Received 20 April 2000/Accepted 21 July 2000)

ABSTRACT. In isolating skeletal muscle satellite cells, sometimes a problem is encountered in removing contaminating nonmyogenic cells. In the present study, we constructed a novel vector, pSKA-EGFP, which achieves the expression of enhanced green fluorescent protein (EGFP) exclusively in myogenic cells under the control of skeletal α-actin promoter when transfected to primary cultured cells from skeletal muscle. Cells from rat skeletal muscle positive for EGFP after transfecting with pSKA-EGFP were all positive for desmin and none of the nonmyogenic cells expressed EGFP, indicating that the expression of EGFP is specific to myogenic cells. Among the cells positive for EGFP were proliferating cells, presumably satellite cells. In addition, EGFP positive cells derived from horse skeletal muscle after transfecting pSKA-EGFP *in vitro* formed multinuclear myotubes, indicating that myogenic expression of EGFP driven by skeletal α-actin was achieved also in the equine cells. These results indicated that pSKA-EGFP vector will be useful in identifying and following up the satellite cells in real time, and also permit us to isolate satellite cells in combination with fluorescence-activated cell sorting (FACS).
KEY WORDS: EGFP, myogenic, satellite cell.

J. Vet. Med. Sci. 62(11): 1213–1216, 2000

Skeletal muscle satellite cells are mononuclear cells located between the basement membrane and the plasma membrane of myofibres in mature muscle [8, 9, 11, 15]. They are mitotically quiescent unless a stimulus is applied to skeletal muscle tissue, but when the skeletal muscle fibres are injured they are activated to proliferate, differentiate, then fuse with each other to form multinuclear myotubes. Satellite cells are also important for postnatal skeletal muscle hypertrophy due to their ability to add new myonuclei into growing myofibres [9, 11, 15] and the number of satellite cells present in the adult skeletal muscle is known to decrease along with the growth of the animal [11]. The establishment of a culture system for satellite cells would assist in understanding the molecular and cellular mechanisms underlying skeletal muscle growth and development but their extrinsic and intrinsic regulations differ among several species [6].

To date, *in vitro* primary culture systems for satellite cells have been described in some species such as pigs [7], cattle [5], rodents [4], humans [3], turkeys [10], and horses [13], but, because of their relative scarcity in adult tissues and of the presence of nonmyogenic cells in primary culture of satellite cells [13], it is sometimes difficult to obtain detailed information regarding the extrinsic and intrinsic regulation of satellite cells with these crude systems. To eliminate the potential paracrine action between myogenic and nonmyogenic cells, clonal analyses of satellite cells have been intended [6] but the optimization of several factors such as culture media, sera and substrata is still a matter of trial and error.

Hoping to solve the problems described above, we constructed a new myogenic vector (pSKA-EGFP) which contains EGFP gene driven by skeletal α-actin promoter [16]. Since skeletal α-actin is known to be expressed almost exclusively in skeletal muscle cells [2, 12], we expected that the EGFP expression would be restricted to myogenic cells when transfecting the primary cultured cells from skeletal muscle which contains both myogenic and nonmyogenic cells. In addition, this approach would allow us to identify and follow the satellite cells in real time, and also permit us to isolate satellite cells in combination with fluorescence-activated cell sorting (FACS). In the present study we transfected cells from the skeletal muscle of rats and horses with pSKA-EGFP vector, and examined whether the EGFP expression would be seen in myogenic cells, including satellite cells.

The plasmid vector, pCX-EGFP, containing the 732 bp fragment of EGFP was kindly provided by Dr. Okabe (Osaka University). pCX-EGFP was digested with EcoRI and the released EGFP fragment was ligated to the EcoRI site of the plasmid vector, pCXN2, kindly provided by Dr. Miyazaki (Osaka University) (pCXN2-EGFP). pCXN2-EGFP was digested with SalI and XbaI to release both CMV-enhancer and chicken α-actin promoter, and then end-filled with Klenow. The pIBI20 plasmid containing the 730 bp fragment of rat skeletal α-actin promoter (a generous gift from Dr. Moshe Shani, Volcani Institute of Agriculture, Rehovot, Israel) [12] was digested with EcoRI and XhoI, and the insert was end-filled with Klenow. The rat skeletal α-actin promoter thus obtained was then inserted into pCXN2-EGFP which lacks both CMV-enhancer and chicken α-actin promoter, and the resultant combination was designated as

* CORRESPONDENCE TO: SOETA, C., M. S., Laboratory of Applied Genetics, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.

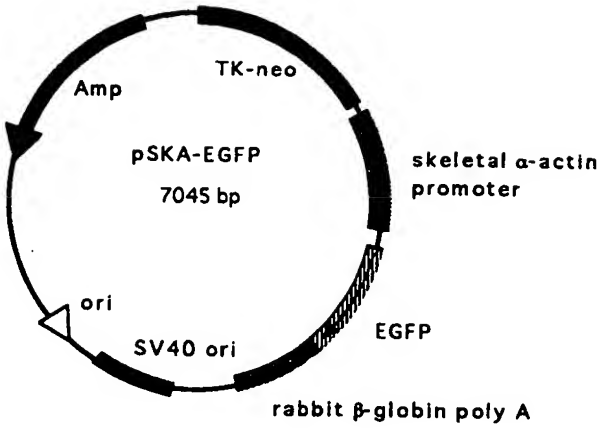


Fig. 1. Schematic representation of the pSKA-EGFP vector.

pSKA-EGFP (Fig. 1).

Adult female Wistar Imamichi rats were purchased from Imamichi Institute of Animal Reproduction (Ibaraki, Japan). Five hundred μl of 20% sodium chloride solution was injected into the femoral muscle of each rat under light ether anesthesia. Forty-eight hours after the injection, the rats were killed by cervical dislocation and the skeletal muscles were removed. The tissues were minced and incubated with Accutase reagent (Innovative Cell Tech., CA) with continuous stirring at 37°C. Dissociated cells were filtered through two layers of lens paper to remove tissue debris and multinucleated cells as well as to enrich mononucleated cells [13]. The cells were resuspended in DMEM containing 10% fetal bovine serum (FBS), 100 U/m*l* penicillin, 100 μg/m*l* streptomycin and 50 μg/m*l* gentamycin, and seeded in a 24-well plate at a density of 5×10^4 cells/well. Transfection was performed on the next day. In the experiment with primary cultured cells from horse skeletal muscles, the cells were isolated from soleus muscle and cultured as previously described [13].

The cells were transfected with 1 μg of the plasmid by Transfast reagent (Promega, WI) according to the method described by the manufacturer. In brief, the cells in each well were incubated with a mixture of DNA-lipid in 200 μl of OPTI-MEM (Gibco-BRL, NY) at 37°C for 1 hr, then 1 m*l* of Dulbecco's modified eagle medium (DMEM) containing 10% FBS and antibiotics was added. EGFP expression was observed under an Olympus photo microscope (B201, Olympus, Tokyo) equipped with epi-illumination and specific filters for fluorescein.

For immunocytochemical analyses, all procedures were done at room temperature. Cells were fixed with 10% formalin in phosphate buffered saline (PBS) for 20 min, then permeabilized with 0.1% Triton X-100 in PBS for 2 min (for staining desmin) or with methanol for 5 min (for staining proliferating cell nuclear antigen, PCNA). After washing in PBS, the cells were blocked in 1% BSA in PBS for 30 min. The primary antibody specific for desmin (DE-U-10, Sigma, MO, at a dilution of 1:100 with 1% BSA in PBS) or PCNA (PC10, Santa Cruz Biotech., CA, at a dilution of 1:100 with 1% BSA in PBS) was applied and incubated for 60 min. As a secondary antibody, rhodamine-labeled goat anti-mouse IgG (Chemicon, CA, diluted at 1:100 with 1% BSA in PBS) was used and incubation was performed for 90 min. Then the specimens were washed with PBS and fluorescein was observed under the microscope.

Mononuclear cells obtained from regenerating rat femoral muscles were transfected with pSKA-EGFP or pCXN2-EGFP, and their EGFP expression was observed. EGFP expression was seen in the cells both transfected with pSKA-EGFP and with pCXN2-EGFP. To examine if these EGFP positive cells are myogenic cells or not, the cells were stained with anti-desmin antibody. The cells transfected with pCXN2-EGFP did not necessarily express desmin, suggest-

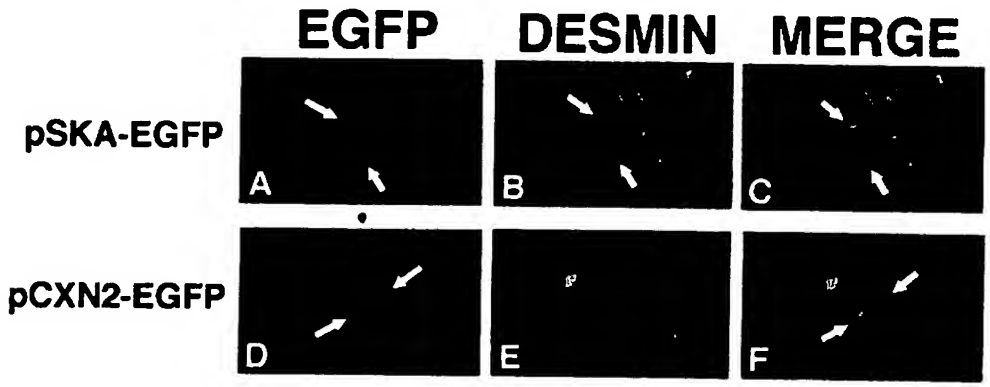


Fig. 2. Myogenic expression of EGFP driven by skeletal α-actin promoter in the primary cultured cells from rat skeletal muscle. Cells obtained from rat skeletal muscle were transfected with pSKA-EGFP or pCXN2-EGFP. Two days after transfection, expression of EGFP was examined and immunocytochemical analysis on the presence of desmin was performed. Note the absence of desmin in EGFP positive cells transfected with pCXN2-EGFP. A and D, EGFP; B and E, desmin; C and F, merged photos. Arrows, corresponding cells.

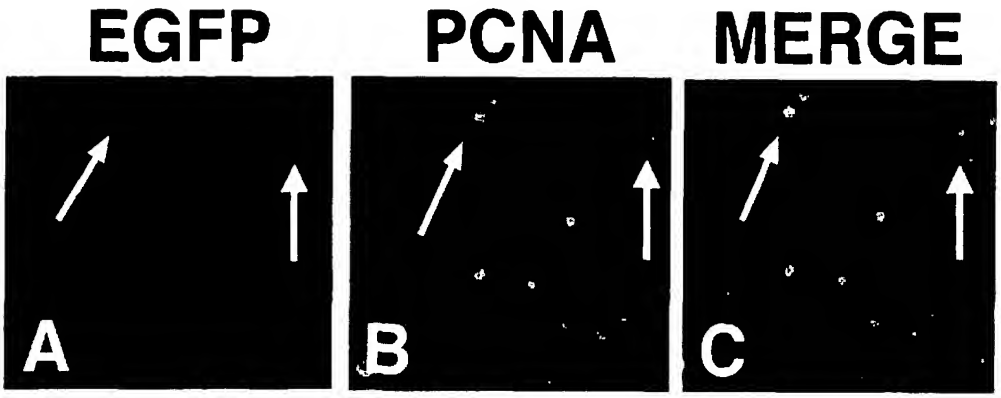


Fig. 3. EGFP expressing cells after transfecting pSKA-EGFP contained proliferating myogenic cells (satellite cells). Cells obtained from rat skeletal muscle were transfected with pSKA-EGFP. Two days after transfection, expression of EGFP was examined and immunocytochemical analysis on the presence of PCNA was performed. A, EGFP; B, PCNA; C, merged photo. Arrows, corresponding cells.

ing that in these cells expression of EGFP was driven ubiquitously (Fig. 2D-F). On the other hand, desmin was expressed in all the EGFP-positive cells transfected with pSKA-EGFP, indicating that skeletal α-actin promoter used to drive the expression of EGFP is activated only in myogenic cells (Fig. 2A-C).

Since the specificity of the pSKA-EGFP to promote myogenic EGFP expression was confirmed, we next examined whether these EGFP positive myogenic cells contained proliferating cells, i. e., satellite cells. Mononuclear cells from rat skeletal muscle were transfected with pSKA-EGFP and immunocytochemical analysis for the presence of PCNA was performed. As shown in Fig. 3, among the cells positive for EGFP are contained cells also positive for PCNA, suggesting that these cells double positive for EGFP/PCNA are satellite cells.

The use of pSKA-EGFP was also applied to the cells from species other than rats. Primary cultured cells from horse soleus muscle were prepared and pSKA-EGFP was transfected. Mononuclear cells positive for EGFP were observed at 2 days after transfection, and when the culture period was extended for another 4 days, some EGFP positive cells fused and formed a multinuclear myotube (Fig. 4). This indicated that pSKA-EGFP could drive myogenic expression of EGFP also in the horse skeletal muscle cells. At 2 days after transfection, the ratio of EGFP positive cells transfected with pSKA-EGFP to those transfected with pCXN2-EGFP within the culture of the horse cells was 1:7. This was in good agreement with the fact that satellite cells constitute 1 to 10% of the total number of cells present in skeletal muscle [11].

A widely used procedure for identifying skeletal muscle satellite cells is staining the cells with antibodies to muscle specific protein(s). Due to their quite similar cell morphology to that of fibroblasts *in vitro*, identifying satellite cells without staining is considerably difficult. One of the well-known markers for satellite cells in several species is an intermediate filament, desmin [1] but procedures for staining cells with antibodies require the fixation of cells and this

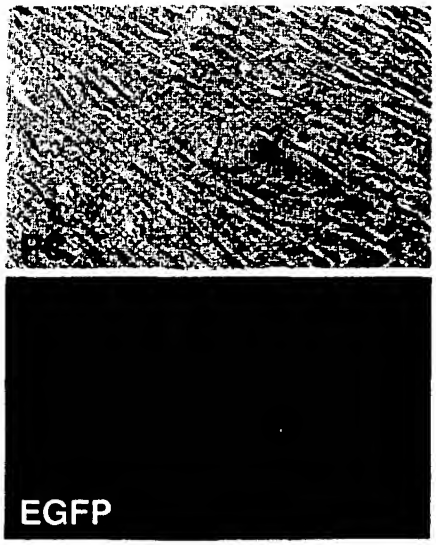


Fig. 4. Formation of multinuclear myotube from pSKA-EGFP transfected horse skeletal muscle cells after 4 days in culture. Culture of horse skeletal muscle derived-cells was transfected with pSKA-EGFP. When the presence of EGFP positive cells was observed, culturing period was extended for more 4 days. Note the formation of EGFP positive multinuclear myotubes. PC, phase contrast.

results in a loss of their viability. A similar strategy to identify a specific cell population by using EGFP expression driven under a promoter, and to observe them in a real time was reported by Wang *et al.* [14]. To identify and isolate neural precursor cells, they linked Tα1 tubulin promoter to the EGFP gene and transfected embryonic rat and chick brain cells with their combination, followed by FACS to enrich neural precursor cells. In analogy, the pSKA-EGFP vector constructed by us in the present study will be a useful tool for

isolating skeletal muscle satellite cells if used in combination with FACS.

This work was supported in part by a grant-in-aid from the Equine Research Institute, Japan Racing Association.

REFERENCES

1. Allen, R. E., Rankin, L. L., Greene, E. A., Boxhorn, L. K., Johnson, S. E., Taylor, R. G. and Pierce, P. R. 1991. *J. Cell Physiol.* 149: 525–535.
2. Asante, E. A., Boswell, J. M., Burt, D. W. and Bulfield, G. 1994. *Transgenic Res.* 3: 59–66.
3. Baroffio, A., Bochaton-Piallat, M., Gabbiani, G. and Bader, C. R. 1995. *Differentiation* 59: 259–268.
4. Bischoff, R. 1974. *Anat. Rec.* 180: 645–662.
5. Dodson, M. V., Martin, E. L., Brannon, M. A., Mathison, B. A. and McFarland, D. C. 1987. *Tissue Cell* 19: 159–166.
6. Dodson, M. V., McFarland, D. C., Grant, A. L., Doumit, M. E. and Velleman, S. G. 1996. *Domest. Anim. Endocrinol.* 13: 107–126.
7. Doumit, M. E. and Merkel, R. A. 1992. *Tissue Cell* 24: 253–262.
8. Husmann, I., Soulet, L., Gautron, J., Martelly, I. and Barritault, D. 1996. *Cytokine Growth Factor Rev.* 7: 249–258.
9. Mauro, A. 1961. Satellite cells of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* 9: 493–501.
10. McFarland, D. C. 1992. *J. Nutr.* 122: 818–829.
11. Schultz, E. and McCormick, K. M. 1994. *Rev. Physiol. Biochem. Pharmacol.* 123: 213–257.
12. Shani, M. 1986. *Mol. Cell. Biol.* 6: 2624–2631.
13. Soeta, C., Yamanouchi, K., Hasegawa, T., Ishida, N., Mukoyama, H., Tojo, H. and Tachi, C. 1998. *J. Equine Sci.* 9: 97–100.
14. Wang, S., Wu, H., Jiang, J., Delohery, T. M., Isdell, F. and Goldman, S. A. 1998. *Nature Biotech.* 16: 196–201.
15. Yablonka-Reuveni, Z. 1995. *Microsc. Res. Tech.* 30: 366–380.
16. Zakut, R., Shani, M., Givol, D., Neuman, S., Yaffe, D. and Nudel, U. 1982. *Nature (Lond.)* 298: 857–859.